Inflammation, local vascular glucocorticoid regulation and endothelial function

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Abstract

Glucocorticoids can act upon vascular cells to alter contractile function, influence structure and modulate the inflammatory response to vascular injury. Tissue-specific glucocorticoid availability is modulated by the isozymes of 11β-hydroxysteroid dehydrogenase which inter-convert active glucocorticoids and their inactive metabolites. 11β-Hydroxysteroid dehydrogenase activity in the vascular wall may contribute to local feedback regulation of inflammation, especially since pro-inflammatory cytokines up-regulate 11βHSD1 and down-regulate 11βHSD2 in cultured human aortic smooth muscle cells. It was hypothesised that inflammatory mediators enhance local glucocorticoid generation by 11β-hydroxysteroid dehydrogenases in intact vascular tissue with resultant impairment of endothelial cell function.

11β-Reductase and dehydrogenase activities were detected in intact mouse aorta and iliofemoral arteries *in vitro*, and in the perfused mouse hindquarter *in vivo*. 11β-reduction was the predominant reaction direction. Use of mice with genetic inactivation of either 11βHSD1 or 11βHSD2 demonstrated that 11βHSD2 acts as an exclusive dehydrogenase. 11βHSD1 exhibited bidirectional activity in intact arteries *in vitro* but was shown to be a predominant reductase *in vivo*. These studies confirm the predominant regeneration of glucocorticoids by the 11β-hydroxysteroid dehydrogenases within the vessel wall and suggest that these isozymes play an important role in modulating intra-vascular glucocorticoid signalling.

11βHSD1 activity in cultured murine aortic smooth muscle cells was up-regulated following incubation with the pro-inflammatory cytokine IL-1β. By contrast, there was no such effect of inflammatory mediators on 11βHSD activity in intact aortic rings *in vitro*. Systemic *in vivo* LPS administration resulted in a modest increase in 11β-reductase activity in aortic rings *ex vivo*, but did not alter 11β-reductase activity in the perfused hindquarter *in situ*. These data suggest that up-regulation of 11βHSD1 reductase is unlikely to be a significant accompaniment of vascular inflammation in healthy arteries *in vivo*. However, the possibility remains that

11βHSD1 is up-regulated in pathological conditions associated with intense cell proliferation, such as vessel injury or atheroma.

Consequent changes in glucocorticoid levels within vessels during inflammation would be relevant as glucocorticoids directly influence many aspects of vascular structure and function, including the vascular response to inflammation and/or injury. To examine the effects of variations in glucocorticoid availability on endothelial cell function, forearm venous occlusion plethysmography studies were conducted in healthy volunteers following acute manipulation of circulating glucocorticoid levels. Short term changes in systemic glucocorticoid concentrations did not significantly alter endothelial cell vasomotor or fibrinolytic function in the human forearm. The explanation for these negative findings may lie in the exquisite 11βHSD system, central to this thesis, whereby, despite fluctuations in circulating glucocorticoid concentrations, vascular intracellular glucocorticoids levels are tightly regulated.

In conclusion, the studies presented in this thesis demonstrate that the isozymes of 11βHSD modulate local glucocorticoid concentrations within intact murine vasculature. However, glucocorticoid metabolism by the 11βHSDs in healthy murine arteries is not altered by inflammatory mediators. Finally, acute systemic variations in glucocorticoid availability do not impair endothelial cell vasomotor or fibrinolytic function in humans *in vivo*.

Declaration

I declare that this thesis was written by me and that the data presented represent my own work, with the exceptions listed below:

Primary murine aortic smooth muscle cell cultures were established and maintained by Eileen Miller of the Centre for Cardiovascular Science, University of Edinburgh. Eileen also performed the cortisol ELISAs.

PAI-1 and t-PA ELISAs were performed by Pamela Dawson of the Centre for Cardiovascular Science, University of Edinburgh.

TNFα bioactivity assays were performed by Carol Ward of the Centre for Inflammation Research, University of Edinburgh.

I declare that this work has not been submitted for any other degree.

Anna Rachel Dover, Edinburgh 2006

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Contents

List of Figures

List of Tables

List of Abbreviations

A	11-Dehydrocorticosterone
ACh	Acetylcholine
ACRD	Apparent cortisone reductase deficiency
ACTH	Adrenocorticotrophic hormone
AII	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AVP	Arginine vasopressin
B	Corticosterone
BH_4	Tetrahydrobiopterin
BK	Bradykinin
BSA	Bovine serum albumin
CBG	Corticosterone binding globulin
CBP	CREB-binding protein
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CREB	cAMP response element binding protein
CRH	Corticotrophin-releasing hormone
CYP	Cytochrome P450
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribo-nucleic acid
DTT	Dithiothreitol
EDHF	Endothelium-derived hyperpolarising factor
EDTA	Ethylene diamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FBF	Forearm blood flow
FCS	Fetal calf serum
FDPs	Fibrin degradation products

GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP	Guanosine triphosphate
H6PDH	Hexose-6-phosphate dehydrogenase
HOSE	Human ovarian surface epithelial
HPA-axis	Hypothalamic-pituitary gland-adrenal gland-axis
HPLC	High performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
HUVEC	Human umbilical vein endothelial cell
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IVC	Inferior vena cava
KHB	Krebs'-Henseleit buffer
KRB	Krebs'-Ringer buffer
L-NMMA	NG-monomethyl-L-arginine
LPS	Lipopolysachharide
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinases
MA-SMCs	Murine aortic smooth muscle cells
MPK-1	MAPK phosphatase-1
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NF-κB	Nuclear factor κB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase

PGI_2	Prostacyclin
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
SAME	Syndrome of apparent mineralocorticoid excess
sGC	Soluble guanylyl cyclase
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
TNF-α	Tumor necrosis factor-α
t-PA	Tissue plasminogen activator
TXA_2	Thromboxane A_2
VSMαA	Vascular smooth muscle α-actin

List of Publications, Presentations and Awards

Reviews

Hadoke PWF, Macdonald L, Logie JJ, Small GR, **Dover AR**, Walker BR. Intravascular glucocorticoid metabolism as a modulator of vascular structure and function. Cellular and Molecular Life Sciences (2006) 63(5):565-78.

Original Research

Dover AR, Hadoke PWF, MacDonald LJ, Miller E, Newby DE, Walker BR. Intravascular glucocorticoid metabolism during inflammation and injury. Submitted to Endocrinology (2006).

Dover AR, Newby DE, Hadoke PWF, Walker BR. Effects of acute manipulation of systemic glucocorticoid concentrations on endothelial fibrinolytic and vasodilator function in humans. Submitted to the Journal of the American College of Cardiology (2006).

Small GR, Hadoke PWF, Sharif I, **Dover AR**, Armour D, Kenyon CJ, Gray GA, Walker BR. Preventing Local Regeneration of Glucocorticoids by 11β-Hydroxysteroid Dehydrogenase Type 1 Enhances Angiogenesis. Proceedings of the National Academy of Sciences USA (2005) 102(34):12165-12170.

Abstracts

Dover AR, Newby DE, Hadoke PWF, Walker BR. Short-term variations in circulating glucocorticoid levels do not influence endothelial vasomotor or fibrinolytic function. American Endocrine Society, Boston, 2006.

Dover AR, Hadoke PWF, Miller E, Newby DE, Walker BR. Inflammatory Cytokines Up-regulate 11β-Hydroxysteroid Dehydrogenase Type 1 in Murine Vascular Smooth Muscle Cells in vitro but not in vivo. American Endocrine Society, San Diego, 2005.

Dover AR, Hadoke PWF, Ogston E, Newby DE, Walker BR. Interleukin-1β Impairs 11β-Hydroxysteroid Dehydrogenase Type 1-Mediated Generation of Glucocorticoids in the Mouse Aorta. Joint meeting of the Scottish Society for Experimental Medicine and Scottish Cardiac Forum, Glasgow 2004.

Dover AR, Ogston E, Hadoke PWF, Newby DE, Walker BR. Generation of Glucocorticoids by 11β-Hydroxysteroid Dehydrogenase Type 1 Within Intact Mouse Aorta is not Enhanced by Proinflammatory Cytokines. 9th Annual Meeting of the European Council for Cardiovascular Research, Nice 2004

Dover AR, Hadoke PWF, Miller E, Newby DE, Walker BR. Generation of Glucocorticoids by 11β-Hydroxysteroid Dehydrogenase Isozymes in the Perfused Mouse Hindlimb. Scottish Society for Experimental Medicine, Edinburgh 2004; Scottish Cardiac Forum, Aberdeen 2005; British Endocrine Society Annual Meeting, Harrogate 2005

Small GR, **Dover AR**, Hadoke PWF, Walker BR. Local Regeneration of Glucocorticoids by 11βHSD-1 Within the Vessel Wall Modulates Angiogenesis. Joint meeting of the Scottish Society of Experimental Medicine and Scottish Cardiac Forum, Glasgow 2004

Small GR, **Dover AR**, Hadoke PWF, Walker BR. Local Regeneration of Glucocorticoids by 11βHSD-1 Within the Vessel Wall Modulates Angiogenesis in vitro and in vivo in Mice. American Endocrine Society, New Orleans 2004

Small GR, **Dover AR**, Hadoke PWF, Kenyon CJ, Seckl JR, Walker BR. Angiogenesis is Modulated by Local Regeneration of Glucocorticoids by 11βHSD-1 in the Vessel Wall in vitro and in vivo in Mice. British Atherosclerosis Society Spring Meeting, Oxford 2004

Oral Presentations

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Chapter One

Introduction

Glucocorticoids may interact directly with cells of the blood vessel wall to contribute to their well-established link with the development of cardiovascular disease. Glucocorticoids can act upon vascular cells to alter contractile function, influence structure and modulate the inflammatory response to injury. Local vascular glucocorticoid availability is regulated by the isozymes of 11β-hydroxysteroid dehydrogenase (11βHSD), which inter-convert active glucocorticoids and their inactive metabolites. It has been suggested that regulation of 11βHSD activity contributes to local feedback regulation of inflammation as pro-inflammatory cytokines alter 11βHSD activity and expression in cultured human aortic smooth muscle cells, favouring increased local glucocorticoid concentrations. However, the influence of cytokines on 11βHSD activity in intact arteries, and the consequences of altered glucocorticoid availability on endothelial function *in vivo* have not been established. The hypotheses that inflammatory mediators regulate local vascular glucocorticoid action through effects on the 11βHSDs and that alterations in glucocorticoid availability influence endothelial function are explored in this thesis.

Given the importance of systemic and local glucocorticoid action on vascular function, a comprehensive understanding of these steroids is required. The following chapter reviews glucocorticoid physiology and pathophysiology with particular emphasis on the importance of metabolism by the 11βHSDs within the blood vessel wall. The direct effects of glucocorticoids on vascular function are then reviewed in detail. Finally, the hypotheses and aims of the thesis are described.

1.1 Glucocorticoids

1.1.1 Hormone structure, synthesis, regulation and metabolism

Glucocorticoids (corticosterone in rodents and cortisol in man), originally named for their effects on carbohydrate metabolism, are members of the steroid hormone family synthesised from the common precursor cholesterol. All steroids hormones are derived from the cyclopentanoperhydrophenanthrene structure comprising a cyclopentane ring and three cyclohexane rings, and the unique properties of each

individual steroid are determined by the presence of different chemical groups at specific positions on the molecule (Figure 1.1).

Glucocorticoids are synthesised from cholesterol in the zona fasciculata (and, to a lesser extent, the zona reticularis) of the adrenal cortex. Steroid biosynthesis within the adrenal cortex is catalysed by a series of cytochrome P450 (CYP) enzymes (Figure 1.2), which reside in the membranes of the endoplasmic reticulum and mitochondria. The major active glucocorticoid in mice is corticosterone, rather than cortisol, as these animals lack the adrenal 17α-hydroxylase enzyme required for cortisol synthesis. Glucocorticoids are not stored in the adrenal gland but are synthesised *de novo* and released when required.

Glucocorticoid synthesis and release is regulated by numerous neuro-endocrine signals (eg physical stress, pro-inflammatory cytokines) which act via the hypothalamic-pituitary-adrenal axis. The hypothalamus, in response to stimulation, releases corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypothalamic-hypophyseal portal capillary system. The stimulation of CRH receptors on corticotrophs within the anterior pituitary gland results in rapid release of adrenocorticotrophic hormone (ACTH) into the systemic circulation. ACTH, formed from the processing of pro-opiomelanocortin (POMC) in the anterior pituitary, is secreted in a pulsatile fashion and acts on the adrenal cortex to stimulate synthesis of glucocorticoids and other adrenocortical steroids (Axelrod & Reisine 1984). Glucocorticoids themselves inhibit ACTH synthesis through inhibition of CRH and AVP synthesis in the hypothalamus and by preventing POMC transcription and processing in the anterior pituitary. In this manner, glucocorticoids provide negative feedback to the hypothalamic-pituitary-adrenal axis to maintain physiological circulating glucocorticoid levels. Glucocorticoid biosynthesis is also subject to diurnal variation, as a results of diurnal changes in ACTH pulse frequency and amplitude, with plasma levels highest just prior to waking and reaching a nadir prior to sleep (Dallman *et al.* 1993).

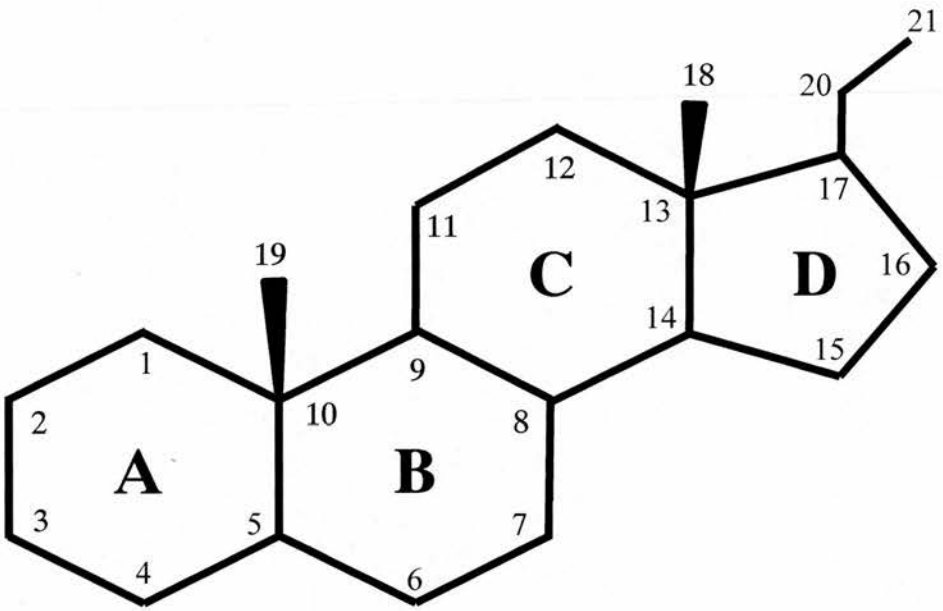

Figure 1.1 Steroid ring structure

The basic steroid ring structure comprising a cyclopentane ring and three cyclohexane rings. Conventional labeling identifies the four carbon rings by letters, and the individual carbon atoms by numbers. Chemical groups are designated according to the number of the carbon atom to which they are attached.

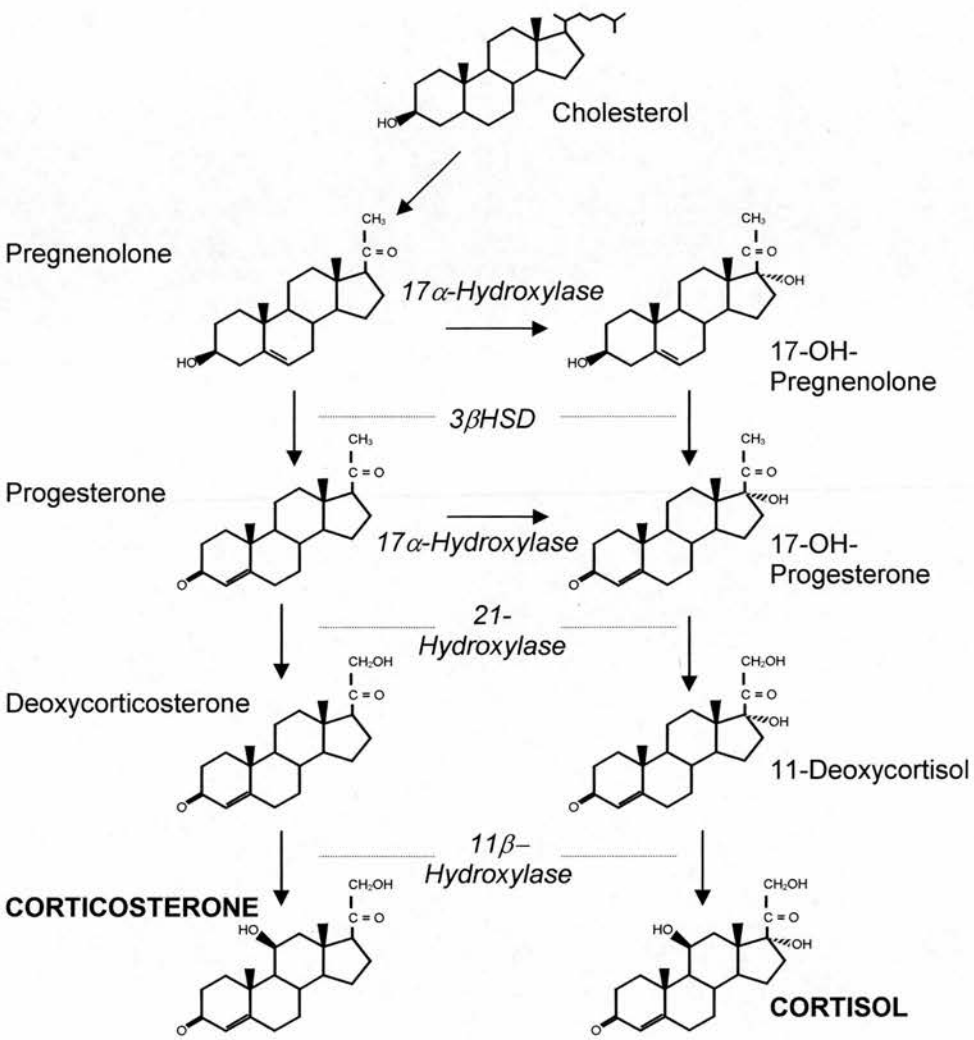

Figure 1.2 Adrenocortical glucocorticoid biosynthesis

Steroid biosynthesis within the adrenal cortex from the precursor cholesterol is catalysed by a series of cytochrome P450 enzymes. The major active glucocorticoid in rodents is corticosterone as they lack adrenal 17α-hydroxylase.

Plasma glucocorticoids are largely found bound to corticosteroid-binding globulin (CBG) and albumin, with only 5-10% circulating in the free unbound state (Hammond *et al.* 1990). Only free steroids are able to diffuse across the capillary basement membrane and cell membrane to bind to the intracellular glucocorticoid receptor, hence these binding proteins act to buffer available glucocorticoid. At high physiological glucocorticoid concentrations, the binding proteins may become saturated and this phenomenon may amplify the diurnal variations in circulating levels of free glucocorticoids.

Inactivation of glucocorticoids is mediated by a complex process of conversion to inactive metabolites in the liver followed by renal excretion. This pathway, illustrated in Figure 1.3, includes inter-conversion with inactive 11-dehydrocorticosterone (or cortisone in man) by the isozymes of 11β-hydroxysteroid dehydrogenase (11βHSD), reduction by 5α/β-reductases, 3α-hydroxysteroid dehydrogenase (3αHSD) and 20α/β-hydroxysteroid dehydrogenases, followed by oxidation by 21-oxidase and, finally, conjugation to either glucuronic acid or sulphates to facilitate urinary excretion. Whilst the 11-keto metabolites formed by 11β-dehydrogenation of glucocorticoids (11-dehydrocorticosterone in rodents and cortisone in man) are biologically inert, some of the other intermediate products of glucocorticoid metabolism (eg 5α-tetrahydrocorticosterone) have the capacity to bind and activate the glucocorticoid receptor (McInnes *et al.* 2004).

1.1.2 Glucocorticoid action

Classical glucocorticoid action is mediated through the binding of glucocorticoids to intra-cellular corticosteroid receptors. There are two distinct cytosolic corticosteroid receptors: glucocorticoid (GR; type II corticosteroid receptors) and mineralocorticoid (MR; Type I corticosteroid receptors) receptors, both of which are members of the steroid/thyroid hormone receptor super family of ligand-activated transcription factors (Parker 1993). Although only one gene for GR has been identified, several isoforms exist (with GRα predominating) as a result of alternative splicing and the use of multiple promoters

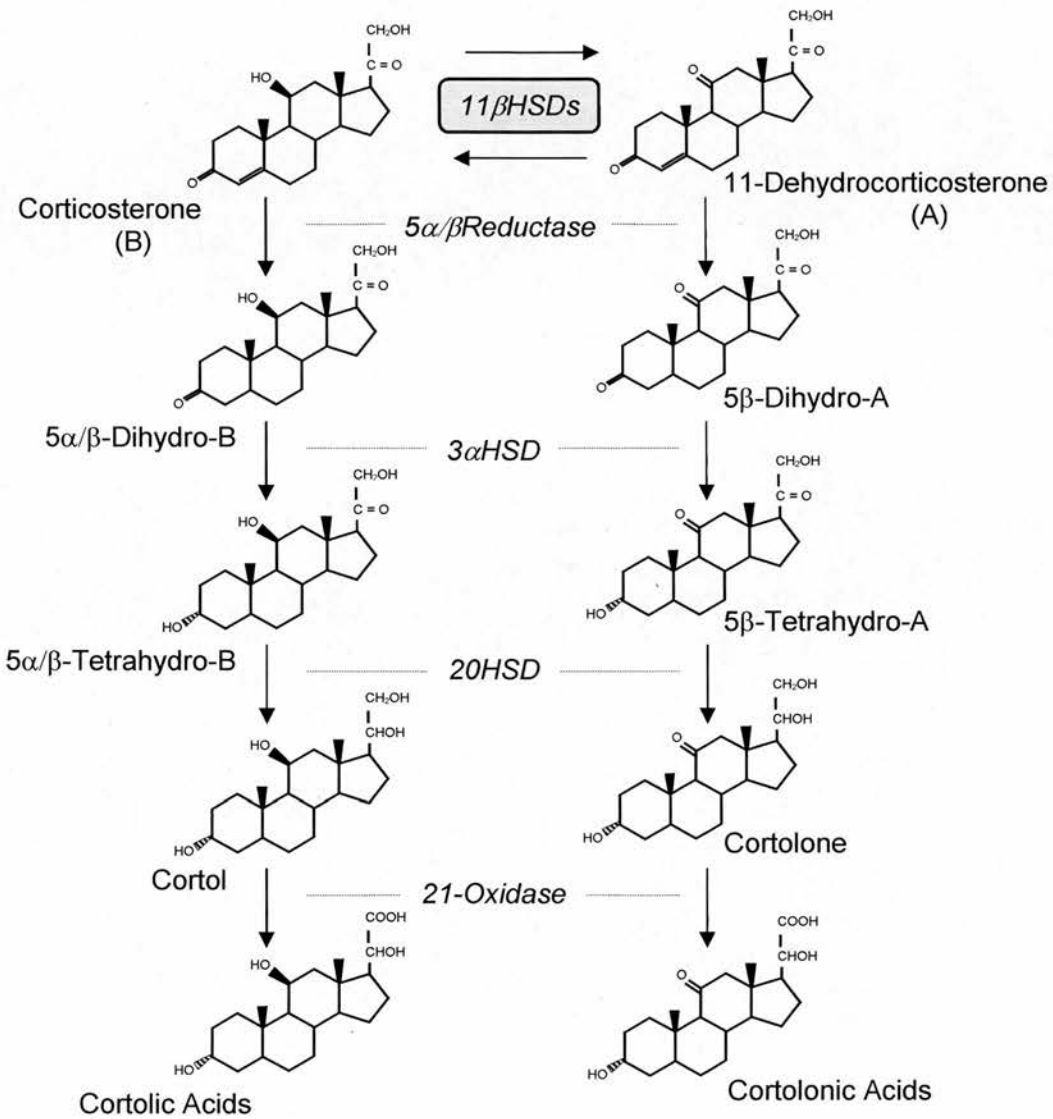

Figure 1.3 Pathway of glucocorticoid metabolism

Glucocorticoids are inactivated through conversion to inactive metabolites by reduction, oxidation, hydroxylation and conjugation. The enzymes catalysing these reactions are indicated. A = 11-dehydrocorticosterone; B = corticosterone; HSD = hydroxysteroid dehydrogenase.

Although glucocorticoids are able to bind to both MR (whose normal ligands are mineralocorticoids, such as aldosterone) and GR, pre-receptor metabolism of glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase prevents illicit occupation of MR in mineralocorticoid sensitive tissues. Thus, physiological glucocorticoid effects are predominantly mediated by binding to GR rather than MR. There are exceptions to this, for example in the hippocampus, where MR is not protected by 11βHSD2 and is activated by both glucocorticoids and mineralocorticoids (Sheppard & Funder 1987b; de Kloet *et al.* 1998).

Following diffusion of unbound glucocorticoid across the capillary basement membrane and across the cell membrane, ligand binding leads to dissociation of GR from inhibitory heat shock proteins, GR receptor phosphorylation and activation, dimerisation and translocation to the nucleus where the glucocorticoid receptor complex binds to specific palindromic DNA sequences known as glucocorticoid response elements (GREs). GREs are located in the promoter region of target genes (Yamamoto 1985) and activation of these elements by activated GR interferes with components of the transcription machinery, leading to stimulation or repression of gene transcription. Activated GR may also indirectly influence gene transcription through cross-talk with other transcription factors, including the pro-inflammatory transcription factors AP-1 and nuclear factor-κB (NF-κB) (Marx 1995). Functional antagonism between GR and subunits of both NF-κB and AP-1 can disrupt pro-inflammatory gene activation and interfere with inflammation-mediated signalling pathways.

In addition to the classical mechanisms of glucocorticoid action, there is increasing evidence that glucocorticoids exert specific "non-genomic" effects. Examples exist of rapid glucocorticoid effects (for example, on phospholipase A_2 and phosphoinositide-3-kinase-mediated eNOS release) that are mediated by GR but are transcription-independent (Limbourg *et al.* 2002; Hafezi-Moghadam *et al.* 2002). Some of the non-genomic effects of glucocorticoids are thought to be mediated by as yet uncharacterised membrane-coupled receptors (Bartholome *et al.* 2004).

1.1.3 Physiological effects of glucocorticoids

Glucocorticoids influence many body systems directly, and also indirectly, though modulation of other hormone systems. They influence metabolic and homeostatic processes as well as exerting effects on the cardiovascular system, the immune system, the central nervous system, the reproductive system, the eye, bone, muscle and during growth and development.

The abundant actions of glucocorticoids first came to light upon identification of phenotypes of adrenocortical excess and insufficiency. Addison's disease, a state of adrenocortical deficiency, is characterised by hypoglycaemia, weight loss, anorexia and postural hypotension (Addison 1855). By contrast, Cushing's syndrome of glucocorticoid excess results in numerous clinical manifestations including depression, central adiposity, insulin resistance, hypertension and dyslipidaemia (Cushing 1912).

1.1.3.1 Effects on metabolism

Glucocorticoids regulate hepatic and peripheral metabolism of carbohydrate, fat and protein. These steroids increase blood glucose concentrations by inhibiting peripheral glucose utilisation, stimulating hepatic gluconeogenesis and reducing insulin secretion. Peripheral glucose uptake is inhibited through repression of translocation of the glucose transporter GLUT4 to the cell membrane (Rizza *et al.* 1982). Gluconeogenesis is stimulated by glucocorticoids as they promote availability of gluconeogenic substrates, by enhancing skeletal muscle catabolism and lipolysis (Exton 1979), and increase the expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK; Sasaki *et al.* 1984). Glycogen production is enhanced by glucocorticoids through increased synthesis by glycogen synthase and reduced metabolism by glycogen phosphorylase (Stalmans & Laloux 1979). Glucocorticoids also alter fat metabolism by stimulating lipolysis, which releases free fatty acids into the circulation, and by promoting differentiation of pre-

adipocytes to adipocytes (Hauner *et al.* 1987). Indeed, glucocorticoid excess is associated with redistribution of body fat from the peripheral to the visceral compartments (Rebuffe-Scrive *et al.* 1988). These various effects of glucocorticoids on carbohydrate and fat metabolism are highly dependent on the caloric state of the individual, however. In general terms, glucocorticoids promote the mobilisation of substrate from peripheral fat and protein stores in times of starvation, through lipolysis and muscle catabolism, whilst fostering the accumulation of abdominal adipose, and hence obesity, under conditions of caloric excess (Dallman *et al.* 2004).

Glucocorticoid excess, by promoting skeletal muscle catabolism, is associated with a proximal myopathy. Additionally, glucocorticoid excess can also cause osteoporosis, by inhibiting intestinal calcium absorption and increasing renal calcium excretion (Canalis 1996), and by inhibiting new bone formation (Manolagas 2000). Finally, glucocorticoids exert adverse effects on connective tissue through inhibition of fibroblast function and extracellular matrix synthesis (Pratt & Aronow 1966).

1.1.3.2 Effects on immunity and inflammation

Glucocorticoids modulate many facets of the immune system and are perhaps best known for their potent anti-inflammatory actions, which account for their most common therapeutic applications. Inflammation is a tightly controlled process (Nathan 2002), involving recruitment and activation of cells of the immune system in response to infection or injury. Cytokines are secreted proteins which regulate almost every aspect of the inflammatory response through effects on cell growth, differentiation and activation (Borish & Steinke 2003). Pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β), produced predominantly by macrophages and antigen presenting cells, bind to cell surface receptors thereby activating multiple interacting signal transduction pathways including the receptor tyrosine kinases, mitogen-activated protein kinases (MAPK), janus kinases and other kinase pathways involved in nuclear factor-κB (NF-κB) activation. Transcription factors such as NF-κB and activator protein-1 (AP-1) then translocate to the nucleus, bind to DNA and induce inflammatory gene transcription.

The final responses to stimulation by the cytokines TNFα and IL-1β include neutrophil and T-lymphocyte activation, and the induction of endothelial cell adhesion molecules to facilitate trafficking of granulocytes to the appropriate site. A potent stimulus for pro-inflammatory cytokine expression is lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls. LPS becomes bound to circulating LPS-binding protein, an acute phase protein, which aids docking of LPS at dimmers of toll-like receptors (TLR4) on the cell surface of macrophages. Activation of TLR4 receptors leads to the initiation of NF-κB, IRF3 and MAPK kinase pathways, complex signalling cascades resulting in expression of many pro-inflammatory cytokines (including TNFα and IL-1β), chemokines and immediate early transcription factors (Wells *et al.* 2005). LPS-induced inflammation is potently suppressed by the cytokines IL-4 and IL-13, secreted by T-helper lymphocytes (Hart *et al.* 1999).

During inflammation, circulating glucocorticoid levels are elevated as a result of HPA axis activation (Munck *et al.* 1984). Inflammatory stimuli such as TNFα and IL-1β stimulate glucocorticoid secretion by enhancing expression of both CRH and ACTH (Turnbull & Rivier 1999), and by promoting production of ACTH secretagogues such as noradrenaline, pituitary adenylate cyclase-activating polypeptide, vasopressin and other cytokines (Chesnokova & Melmed 2002). Within specific tissues, intracellular glucocorticoid availability may also be enhanced as a result of the effects of inflammatory cytokines on the 11β-hydroxysteroid dehydrogenases (as discussed in depth in Section 1.2.7). In a classical endocrine negative feedback loop, glucocorticoids then act to interrupt the pro-inflammatory cytokine-mediated signalling pathways and alter immune cell function thereby aiding the resolution of inflammation.

The major mechanism by which glucocorticoids inhibit cytokine-mediated signalling cascades is through reducing expression (transrepression) of inflammatory genes such as adhesion factors, cytokines and chemokines. Glucocorticoids attenuate the ability of pro-inflammatory transcription factors such as NF-κB and AP-1 to induce gene expression (transactivation). The antagonistic effects of glucocorticoids on

inflammatory gene transactivation by these transcription factors have largely been attributed to direct protein-protein interactions between activated GR and subunits of NF-κB (p65) (Ray & Prefontaine 1994; McKay & Cidlowski 1998) and AP-1 (c-Jun and c-Fos) (Pfahl 1993), although GR is also able to repress AP-1 by interfering with c-Jun N-terminal kinase activity (Bruna *et al.* 2003). Additionally, glucocorticoids promote the expression of IκBα, the cytoplasmic inhibitor of NF-κB, thereby reducing the amount of NF-κB available to translocate to the nucleus (Auphan *et al.* 1995; Scheinman *et al.* 1995). Alternative mechanisms by which glucocorticoids antagonise the effects of pro-inflammatory transcription factors include inhibition of histone acetylation (which results in tighter coiling of DNA and reduced access of transcription factors to their binding sites) (Ito *et al.* 2000; Adcock *et al.* 2004) and competition for transcriptional co-activators (such as cAMP response element binding protein (CREB)-binding protein (CBP))(Smoak & Cidlowski 2004). Glucocorticoids are also able to directly activate transcription of a number of anti-inflammatory proteins (including lipocortin-1, interleukin 10 and IL-1 receptor antagonist) by classical binding of GR to GREs on DNA (Barnes 1998) and, through transcriptional induction of MAPK phosphatase-1 (MPK-1), may regulate inflammatory signalling cascades by inhibiting phosphorylation/activation of a number of essential kinases (De Bosscher *et al.* 2003).

There is clearly considerable cross-talk between the multiple inflammatory signalling pathways and glucocorticoid receptor activation (Adcock & Caramori 2001), which is unsurprising considering the direct physical interactions between transcription factors, GR and other transcriptional coactivators. Both NF-κB (McKay & Cidlowski 1998) and AP-1 (Periyasamy & Sanchez 2002) are able to directly antagonise GR-mediated gene transcription, and, as mentioned earlier, this mutual antagonism may be due to competition for cofactors such as CBP (Smoak & Cidlowski 2004). The activity of the glucocorticoid receptor, and hence its ability to transactivate GR-dependent genes, also depends upon its phosphorylation state. Kinases involved in the cytokine signalling pathways (such as c-jun N-terminal kinases) can alter the phosphorylation of GR and in doing so inhibit GR-dependent gene transcription (Rogatsky *et al.* 1998).

In addition to their effects on cytokine-mediated signalling pathways, glucocorticoids also modulate the function of the cellular component of the immune system. They alter the peripheral leukocyte differential by increasing circulating granulocytes and reducing circulating monocytes, lymphocytes and eosinophils. Glucocorticoids also have direct actions on both T and B lymphocytes, including inhibition of immunoglobulin synthesis and induction of lymphocyte apoptosis. Synthetic glucocorticoids also contribute to the resolution of inflammation by accelerating the acquisition of phagocytic capacity for apoptotic leukocytes in maturing monocytes and increasing the capacity of individual macrophages to ingest multiple apoptotic cells (Liu *et al.* 1999; Giles *et al.* 2001).

1.1.3.3 Effects on the cardiovascular system

Glucocorticoids exert their influences on cardiovascular physiology and pathophysiology through both systemic and local mechanisms. A comprehensive discussion of the direct vascular effects of glucocorticoids is provided in Section 1.3.

Glucocorticoids play a key role in the regulation of blood pressure. As discussed earlier in Section 1.1.3, glucocorticoid deficiency and excess are associated with hypotension and hypertension, respectively. The mechanisms by which glucocorticoids influence blood pressure are undoubtedly complex, and not fully characterised. Glucocorticoids enhance sensitivity to vasopressors such as norepinephrine and angiotensin II (Sato *et al.* 1994) and impair nitric oxide-mediated endothelial vasodilatation (Mangos *et al.* 2000). Glucocorticoids influence renal electrolyte and water homeostasis via effects on glomerular filtration rate, proximal tubular epithelial sodium transport and free water clearance (Marver 1984). The regulation of intra-vascular volume is also affected by glucocorticoid-dependent release of hepatic angiotensinogen (Saruta *et al.* 1986), hypothalamic AVP (Raff *et al.* 1987) and atrial natriuretic peptide (ANP) from cardiac myocytes. Finally, there may be central nervous system effects of steroids which contribute to the development of hypertension (Scoggins *et al.* 1989).

In addition to their effects on blood pressure, glucocorticoid excess is associated with atherosclerosis. It is now widely recognised that atherosclerosis is an inflammatory disease process (Ross 1999; Libby *et al.* 2002); chronic low-grade inflammation is thought to contribute to the progression of atherosclerotic lesions and inflammatory markers predict those patients at risk of future cardiovascular events (Albert *et al.* 2003; Schwartz *et al.* 2003). The potential therapeutic applications of glucocorticoids, as inhibitors of both inflammation and proliferation, in the treatment of atherosclerosis have been explored. Glucocorticoids inhibit macrophage and lipid accumulation into atherosclerotic lesions (Asai *et al.* 1993; Naito *et al.* 1992), and dexamethasone treatment inhibits neointimal proliferation in some (Villa *et al.* 1994; Guzman *et al.* 1996; Petrik *et al.* 1998; Van Put *et al.* 1995), but not all (Karim *et al.* 1997; Lincoff *et al.* 1997), animal models of vessel injury. Local and systemic glucocorticoid administration also reduces the development of stenoses following vascular stent placement in animals (Strecker *et al.* 1998; Pires *et al.* 2005) and in humans (Versaci *et al.* 2002; Patti *et al.* 2005), although this is in conjunction with adverse vascular morphological changes (Pires *et al.* 2005).

Overall, however, the benefits of glucocorticoids have been disappointing, and may have been offset by their systemic side effects. Moreover, despite their potent anti-inflammatory properties, systemic endogenous or exogenous glucocorticoid excess actually contributes to many of the risk factors for ischaemic heart disease including obesity, hypertension, insulin resistance and dyslipidaemia (Etxabe & Vazquez 1994). In healthy subjects, enhanced cortisol production rates are associated with higher blood pressure, obesity, insulin resistance and impaired glucose tolerance (Walker *et al.* 1998). Furthermore, there is an increased risk of cardiovascular events in patients exposed to exogenous glucocorticoid therapy (Wei *et al.* 2004; Souverein *et al.* 2004). Additionally, there is evidence that glucocorticoids modulate factors involved in coagulation (Brotman *et al.* 2005) and endogenous fibrinolysis (thrombus dissolution) (Udden *et al.* 2002) to produce a pro-thrombotic state. These adverse consequences of systemic glucocorticoid administration have prevented their therapeutic application in the management of atherosclerosis and its sequelae.

However, it is increasingly recognised that local availability of glucocorticoids at the tissue level, rather than circulating concentrations, may determine glucocorticoid action. This concept will be discussed in depth in the following sections.

1.2 11β-Hydroxysteroid dehydrogenases

This section describes the enzymology, physiology and significance of the 11β-hydroxysteroid dehydrogenases, which are crucial determinants of the intra-vascular effects of glucocorticoids.

1.2.1 Overview

The 11β-hydroxysteroid dehydrogenases (11βHSDs) interconvert active glucocorticoids (cortisol in man, corticosterone in rodents) with their inert 11-keto metabolites (cortisone and 11-dehydrocorticosterone, respectively). Tissues which express 11βHSDs can therefore regulate local exposure to active glucocorticoids. Whilst circulating glucocorticoid concentrations are under the control of the hypothalamic-pituitary-adrenal axis (see Section 1.1.1), it is now recognised that the 11β-hydroxysteroid dehydrogenases are key determinants of tissue-specific glucocorticoid hormone action.

1.2.2 History of 11β-hydroxysteroid dehydrogenase

The inter-conversion of glucocorticoids with their inert 11-keto metabolites was first described in 1953 (Amelung *et al.* 1953). Evidence for the importance of this inter-conversion first emerged with the use of cortisone as a potent anti-inflammatory therapy in patients with rheumatoid arthritis (Ward *et al.* 1951). It was not known at the time, but cortisone was in fact the inactive hormone, and 11βHSD activity in the liver was responsible for generation of the active glucocorticoid, cortisol. Studies soon followed which identified 11βHSD activity in placenta (Osinski 1960), liver (Jenkins 1966), and kidney (Bush 1969), though the equilibrium "set point" varied with tissue type – predominantly oxidative (and producing inactive 11-keto

metabolites) in kidney and placenta, and reductive (regenerating active glucocorticoids) in liver. Subsequent isotopic studies (Hellman *et al.* 1971), and studies in patients with renal disease (Srivastava *et al.* 1973; Whitworth *et al.* 1989) revealed that the kidney was a key site for glucocorticoid inactivation by 11βHSD. Glucocorticoid reactivation, by contrast, was found to occur in the liver, as suggested by high cortisol:cortisone ratios in hepatic venous blood (Walker *et al.* 1992a). The opposing enzyme directionalities in liver and kidney have since been attributed to the existence of two distinct isoforms of 11βHSD: a predominantly reductive NADP-dependent type 1 isozyme (11βHSD1) and an NAD-dependent oxidative type 2 isozyme (11βHSD2).

The clinical importance of the 11βHSDs came to light with the identification of the "syndrome of mineralocorticoid excess" (SAME) (Ulick *et al.* 1979; Stewart *et al.* 1988). Patients, predominantly children, presented with signs of mineralocorticoid excess with severe hypertension, sodium retention, hypokalaemia and suppressed renin levels despite low plasma aldosterone concentrations. A similar clinical picture of primary hyperaldosteronism had been noted some time previously in patients receiving carbenoxolone for peptic ulcer disease, or following excessive consumption of liquorice (Epstein *et al.* 1977): carbenoxolone is the hemi-succinate derivative of glycyrrhetinic acid, the active ingredient of liquorice. These conditions were ameliorated by treatment with dexamethasone or the mineralocorticoid antagonist spironolactone (Hoefnagels & Kloppenborg 1983; Shackleton *et al.* 1980; Doll *et al.* 1968) and exacerbated by physiological doses of cortisol (Oberfield *et al.* 1983), suggesting that the pathophysiology was attributable to an ACTH-dependent MR agonist. Patients with SAME, and healthy volunteers treated with glycyrrhetinic acid were also noted to have abnormalities of glucocorticoid metabolism, with elevated urinary free cortisol levels, higher ratios of cortisol:cortisone metabolites and impaired elimination of [^{3}H]-cortisol (Stewart *et al.* 1988; MacKenzie *et al.* 1990). The discovery that liquorice inhibited 11βHSD (Stewart *et al.* 1987; MacKenzie *et al.* 1990; Stewart *et al.* 1990; Monder *et al.* 1989), together with evidence that glucocorticoids co-localised with MR in the kidney following administration of liquorice derivatives (Edwards *et al.* 1988) and were able to

activate the receptor (Souness & Morris 1989), led to the description of the pathology underlying SAME, and explained the paradox of mineralocorticoid receptor selectivity for mineralocorticoids in the presence of glucocorticoids. Unlike the ubiquitously expressed glucocorticoid receptor, the mineralocorticoid receptor, which binds aldosterone and glucocorticoids with equal affinity (Krozowski & Funder 1983; Arriza *et al.* 1987), is localised to aldosterone-target tissues such as the distal nephron and colon (Lombes *et al.* 1990). In these tissues, despite far higher circulating concentrations of glucocorticoids (Sheppard & Funder 1987a), only aldosterone binds MR, due to inactivation of glucocorticoids by 11βHSD2. Hence, inactivation of 11βHSD2 by mutation, as in patients with congenital SAME (Stewart *et al.* 1996), or by inhibition by liquorice (or its derivatives) (Edwards *et al.* 1988; Funder *et al.* 1988) allows illicit activation of MR by glucocorticoids with the resultant syndrome of apparent mineralocorticoid excess. This "apparent mineralocorticoid excess" phenotype is also evident in mice with genetic inactivation of 11βHSD2 which show hypertension associated with hypokalaemia, hypochloraemia, and supressed plasma aldosterone and renin activity (Kotelevtsev *et al.* 1999; Holmes *et al.* 2001). 11βHSD2 was thus identified as the "guardian" of MR, preventing illicit activation by glucocorticoids to permit aldosterone-driven activation (as illustrated in Figure 1.4).

1.2.3 Two isozymes of 11β-hydroxysteroid dehydrogenase

Evidence for the existence of more than one isozyme of 11βHSD came from a number of sources. Studies in the rat kidney showed that 11βHSD did not always co-localise with the mineralocorticoid receptor (Edwards *et al.* 1988; Castello *et al.* 1989; Rundle *et al.* 1989), suggesting that there was an isozyme of 11βHSD which was distinct from that "guarding" MR. Kinetic studies using 11βHSD isolated from liver also suggested that the enzyme would be unlikely to compete with MR for glucocorticoid binding as the Km for 11βHSD was in the μM range (Lakshmi & Monder 1985) whilst the Kd for MR is subnanomolar (Arriza *et al.* 1987).

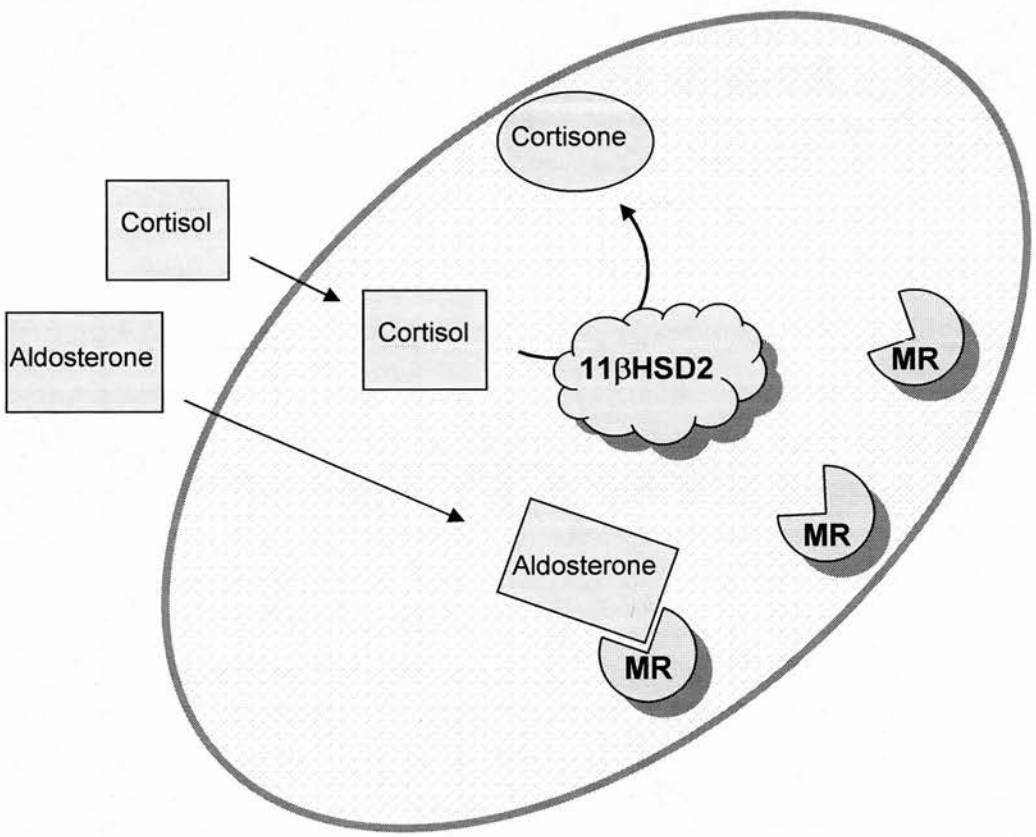

Figure 1.4 Pre-receptor metabolism of glucocorticoids by 11βHSD2

Active glucocorticoids (cortisol in man, corticosterone in rodents) are inactivated by the type 2 isozyme of 11β-hydroxysteroid dehydrogenase. In this manner, illicit occupation of the mineralocorticoid receptor (MR) by glucocorticoids is prevented, thus conferring aldosterone specificity on the receptor.

Clinical evidence for distinct isozymes came from the observation that patients with 11βHSD deficiency had residual 11β-reductase activity and were able to convert oral cortisone to cortisol (Ulick *et al.* 1979; Stewart *et al.* 1988). Furthermore, whilst the 11βHSD inhibitor carbenoxolone inhibited both 11β-reductase and dehydrogenase activity (Stewart *et al.* 1987; Stewart *et al.* 1988), glycyrrhetinic acid only inhibited 11β-dehydrogenase activity (Stewart *et al.* 1990). The conclusive proof of the existence of two isozymes has come with the the cloning of genes for 11βHSD types 1 and 2. 11βHSD type 1 (11βHSD1) was cloned from rat liver in 1989 (Agarwal *et al.* 1989) and the confirmation of a second isoform came with the cloning of 11βHSD type 2 (11βHSD2) from sheep and human kidney (Agarwal *et al.* 1994; Albiston *et al.* 1994) and from human placenta (Brown *et al.* 1996) in the mid 1990s.

1.2.4 11β-Hydroxysteroid dehydrogenase type 2

11βHSD2 is a high affinity exclusive dehydrogenase, catalysing the conversion of glucocorticoids (corticosterone, cortisol) to their inactive 11-keto metabolites (11-dehydrocorticosterone and cortisone, respectively). The gene for 11βHSD2, located on chromosome 16 in humans and chromosome 8 in mice, has 77% sequence homology between species and is therefore highly conserved (Krozowski *et al.* 1999). 11βHSD2 is a microsomal membrane-bound enzyme (Naray & Fejes-Toth 1996), which uses nicotinamide adenine dinucleotide (NAD) as its cofactor and has a Km in the nanomolar range (Brown *et al.* 1993). The primary role of 11βHSD2 is to prevent illicit occupation of MR by glucocorticoids in aldosterone-target tissues such as the distal nephron, sweat glands, salivary glands and colon (Stewart & Krozowski 1999). This concept is supported by the syndrome (SAME) which results from pharmacological inhibition (Monder *et al.* 1989), genetic inactivation (Kotelevtsev *et al.* 1999) or congenital deficiency (Ulick *et al.* 1979; Stewart *et al.* 1988; Mune *et al.* 1995; Wilson *et al.* 1995) of 11βHSD2.

11βHSD2 is also highly expressed in the placenta (Stewart *et al.* 1995; Waddell *et al.* 1998) where it protects the foetus from exposure to excessive maternal glucocorticoids (Brown *et al.* 1996). This is of great importance, as 11βHSD2

inhibition, or maternal dexamethasone administration (dexamethasone is a poor substrate for 11βHSD2), results in low birth weight offspring (Benediktsson *et al.* 1993; Lindsay *et al.* 1996) which may "programme" individuals to a propensity for hypertension, insulin resistance and hypercortisolaemia in adult life (Nyirenda *et al.* 1998; Nyirenda & Seckl 1998).

Our understanding of the physiological importance of the 11βHSDs has been vastly aided by transgenic mouse studies (Paterson *et al.* 2005; Kershaw *et al.* 2005; Kotelevtsev *et al.* 1999; Kotelevtsev *et al.* 1997; Masuzaki *et al.* 2001; Morton *et al.* 2004a). Mice with adipose specific over-expression of human 11βHSD2 (under the control of the murine aP2 promotor; aP2-h11βHSD2) (Kershaw *et al.* 2005) resist weight gain on high-fat diet and have improved glucose tolerance and insulin sensitivity. aP2-h11βHSD2 mice have a favourable adipocytokine profile, with decreased expression of leptin and resistin and increased expression of adiponectin. These data suggest that inactivation of glucocorticoids exclusively in adipose tissue is an important determinant of a favourable metabolic phenotype. By contrast, genetic inactivation of 11βHSD2 is associated with reduced viability and a severe hypertensive phenotype (Kotelevtsev *et al.* 1999), although this phenotype is milder on a C57B6J background than the original MF1 strain. The role of 11βHSD2 in the control of vascular function and blood pressure will be discussed in detail in section 1.3.2.

1.2.5 11β-Hydroxysteroid dehydrogenase type 1

In contrast to 11βHSD2, 11βHSD1 is a low affinity NADP-dependent enzyme expressed in many glucocorticoid target tissues; particularly those which are metabolically active such as adipose and liver (Stewart & Krozowski 1999). The gene for 11βHSD1 is located on chromosome 1 in both humans and mice, and is highly conserved between species, with 85% sequence homology (Krozowski *et al.* 1999). Initially, it was thought that 11βHSD1 played a similar role to that of 11βHSD2 as it acts as a dehydrogenase, inactivating glucocorticoids, in tissue homogenates and microsomes (Lakshmi & Monder 1988). However, it is now

generally accepted that 11βHSD acts as a predominant reductase *in vivo*, regenerating active glucocorticoids. The reductase activity of 11βHSD1 has a K_m of ~1μM *in vitro* (Agarwal *et al.* 1990) (Pu & Yang 2000; Shafqat *et al.* 2003), significantly higher than endogenous concentrations of its substrates, 11-dehydrocorticosterone and cortisone, which circulate in the low nanomolar range (Harris *et al.* 2001). However, 11βHSD1 is now known to occur as a dimeric enzyme (Zhang *et al.* 2005a) which exhibits co-operative kinetics for 11-oxoreduction (Maser *et al.* 2002), permitting dynamic adaptation in response to wide fluctuations in endogenous glucocorticoid levels.

A novel insight into the bi-directional capability of 11βHSD1 has come from the discovery that 11βHSD1 co-localises with hexose-6-phosphate dehydrogenase (H6PDH) within the lumen of the endoplasmic reticulum (ER) (Atanasov *et al.* 2004). H6PDH catalyses the first two steps of the pentose phosphate pathway in which glucose-6-phosphate is utilised to generate nicotinamide adenine dinucleotide phosphate (NADP), the requisite co-factor for 11βHSD1 reductase activity. Co-expression of H6PDH and 11βHSD1 in intact cells results in up-regulation of 11β-reductase activity and down-regulation of 11β-dehydrogenase activity (Atanasov *et al.* 2004; Bujalska *et al.* 2005). Conversely, 11β-dehydrogenation becomes the predominant reaction direction when H6PDH silencing RNA is transfected into cells already expressing 11βHSD1 (Bujalska *et al.* 2005). Furthermore, there is a positive correlation between H6PDH mRNA levels and 11βHSD1 activity, but not mRNA, in human omental preadipocytes (Bujalska *et al.* 2005). These data suggest that H6PDH activity may directly determine the reaction direction of 11βHSD1. Evidence of an interconnection between the 11βHSD1 and H6PDH enzyme systems also comes from findings that glucose-6-phosphate stimulates 11βHSD1 reductase activity in intact microsomes, whilst substrates for 11βHSD1 reductase (11-dehydrocorticosterone or cortisone) or dehydrogenase (corticosterone) activity either enhance or inhibit pentose flux, respectively (McCormick *et al.* 2005; Banhegyi *et al.* 2004). Therefore, the predominant 11β-reduction of steroids by 11βHSD1 *in vivo* has been attributed to its physical proximity to the H6PDH co-factor generating system, and the observation of dehydrogenase activity in tissues *in vitro* (Jellinck *et al.* 1999;

Brem *et al.* 1995) may reflect a change in directionality of 11βHSD1 upon liberation of the enzyme from its intracellular environment (where physical separation of the enzyme from H6PDH favours 11β-dehydrogenation) (Hewitt *et al.* 2005).

Interestingly, congenital 11βHSD1 deficiency, apparent cortisone reductase deficiency (ACRD), first described in 1984 (Taylor *et al.* 1984) and reported in only 11 cases, is associated with few symptoms or signs relating to altered local glucocorticoid metabolism, although there is evidence of increased metabolic clearance of cortisol. The predominant clinical consequences of this deficiency (hirsutism, oligomenorrhoea and acne) relate to impaired negative feedback of cortisol on the HPA axis, resulting in increased ACTH secretion and consequent adrenal androgen excess. The genetic explanation for ACRD is under debate: whilst common polymorphisms in both the 11βHSD1 and the hexose-6-phosphate dehydrogenase (H6PDH) genes have been found together in ACRD patients (Draper *et al.* 2003), these polymorphisms are common (7%) in the general population, and are not associated with features of ACRD (White 2005).

11βHSD1 is thought to be important as an amplifier of glucocorticoid action in glucocorticoid target tissues, and is widely expressed, most notably in liver, adipose tissue, vasculature and the central nervous system. The physiological role of 11βHSD1 in different tissues is discussed in the following sections.

1.2.5.1 11βHSD1 in liver and adipose tissue

11βHSD1 is expressed in both liver (Ricketts *et al.* 1998b), and adipose tissue (Lindsay *et al.* 2003) and its physiological importance in the regulation of metabolism has been elucidated through a series of transgenic studies (Paterson *et al.* 2005; Kotelevtsev *et al.* 1997; Morton *et al.* 2004a; Masuzaki *et al.* 2001). Mice with genetic inactivation of 11βHSD1 appear to have a "cardioprotective" phenotype; they are protected from obesity (Morton *et al.* 2004a), resist stress- and obesity-induced hyperglycaemia (Kotelevtsev *et al.* 1997), have lower serum triglycerides and have a favourable adipocytokine profile, with reduced intra-adipose TNFα and

resistin and increased adiponectin levels. Systemic administration of a novel pharmacological inhibitor of 11βHSD1 to diet-induced obese mice produces a similar improvement in metabolic parameters (Hermanowski-Vosatka *et al.* 2005). Furthermore, 11βHSD1 inhibition in humans results in improved hepatic insulin sensitivity (Walker *et al.* 1995a) and lowers cholesterol (Andrews *et al.* 2003).

Murine adipose over-expression of 11βHSD1 under the tissue-specific promoter aP2 results in a phenotype which mimics that of the metabolic syndrome with obesity, insulin resistance, glucose intolerance, elevated circulating free fatty acids and triglycerides and hypertension despite normal circulating corticosterone levels (Masuzaki *et al.* 2001; Masuzaki *et al.* 2003). Hepatic 11βHSD1 over-expression, under the control of the ApoE promoter has a similar, if less severe, metabolic phenotype but without the obesity and glucose intolerance (Paterson *et al.* 2004). In human idiopathic obesity there is reduced hepatic 11βHSD1 activity (Stewart *et al.* 1999) whilst 11βHSD1 activity in subcutaneous adipose is increased (Rask *et al.* 2001; Rask *et al.* 2002).

1.2.5.2 11βHSD1 in cells of the immune system

The local metabolism of glucocorticoids in tissues of the immune system was first noted in 1960 (Dougherty *et al.* 1960). Subsequent studies have confirmed the presence of 11βHSD1 activity in homogenised preparations of spleen and lymph nodes (Hennebold *et al.* 1996). More recent studies have started to tease apart the cell-specific distribution and role of 11βHSD1. 11βHSD1 mRNA has been detected in mouse (Zhang *et al.* 2005b) and human (Zhou *et al.* 1998) lymphocytes. Interestingly, although 11βHSD1 is not expressed in human monocytes, it is induced upon differentiation to macrophages (Thieringer *et al.* 2001). 11βHSD1 clearly has an important function in these cells, as mice with transgenic inactivation of 11βHSD1 show a delay in the acquisition of macrophage phagocytic capacity and impaired clearance of apoptotic neutrophils (Gilmour JS *et al.*, unpublished observations).

1.2.5.3 11βHSD1 in the central nervous system

11βHSD1 is expressed in both human (Sandeep *et al.* 2004) and rat (Moisan *et al.* 1990; Lakshmi *et al.* 1991) cerebellum, hippocampus and cortex regions of the brain where it may act to modulate the biological effects of glucocorticoids on neuronal development and function. Indeed, 11βHSD1 inhibition, in both man (Sandeep *et al.* 2004) and mouse (Yau *et al.* 2001), also improves cognitive function putatively through lowering glucocorticoid levels in the CNS, where glucocorticoids cause memory impairment.

1.2.5.4 11βHSD1 in other tissues

11βHSD1 is also expressed in a number of other tissues including lung, kidney, colon, fetoplacental, gonad, bone, eye and in malignant cells. A detailed discussion of the function of 11βHSD1 in these tissues is beyond the scope of this thesis and is comprehensively reviewed by Tomlinson *et al.* (2004).

The distribution and physiological importance of 11βHSD1 in vascular tissue is discussed in detail in Section 1.3.2.

1.2.6 Regulation of 11β-hydroxysteroid dehydrogenases

There is complex regulation of the 11βHSDs, much of which has still to be characterised, particularly as methodological difficulties in attributing enyzme activity to each individual isozyme has hindered attempts to study their regulation. As already discussed, tissue-specific activity of the 11βHSDs is central to their role in mediating local glucocorticoid action. Furthermore, studies of the ontogeny of the 11βHSDs, in several tissues and from a number of species, suggests that there is an increase in 11βHSD1 expression during gestation, with a further increase during the pre-pubertal period (Hundertmark *et al.* 1994; Diaz *et al.* 1998; Moisan *et al.* 1992; Maser *et al.* 1994; Yang *et al.* 1992). These data imply that the 11βHSDs are regulated rather than constitutive. Sexual dimorphism of the 11βHSDs has also been

observed, with higher 11βHSD activity in the male rat liver and kidney (Lax *et al.* 1978; Smith & Funder 1991), and in male mouse aorta (Christy 2003) compared with females. There are also some data regarding the sexual dimorphism of 11βHSDs in humans, although the findings have been less consistent (Andrew *et al.* 1998; Stewart *et al.* 1999; Finken *et al.* 1999; Fraser *et al.* 1999).

Increasingly, other factors are being identified which regulate 11βHSD activity and/or expression. These include cytokines, endogenous and synthetic glucocorticoids, growth factors, insulin, sex steroids, thyroid hormones, gonadotrophins, peroxisome proliferators-activated receptor (PPAR) agonists, CRH and ACTH (and are comprehensively reviewed in Tomlinson *et al.* 2004). Glucocorticoids up-regulate 11βHSD1 *in vitro* in rat vascular smooth muscle cells (Takeda *et al.* 1994c), human fibroblasts (Sun & Myatt 2003; Hammami & Siiteri 1991) and rat hepatocytes (Jamieson *et al.* 1995; Liu *et al.* 1996). *In vivo*, 11βHSD1 in the rat liver is induced by glucocorticoids (Low *et al.* 1994) and inhibited by adrenalectomy (Walker *et al.* 1994b). Metyrapone, an inhibitor of 11β-hydroxylase used as a treatment for systemic glucocorticoid excess (Nieman 2002), also inhibits 11βHSD1 in sheep liver microsomes (Sampath-Kumar *et al.* 1997). Peroxisome proliferator-activated receptors (PPARs) are key regulators of glucose and lipid homeostasis, and PPAR agonists are increasingly used for treatment of diabetes mellitus (PPARγ) and hyperlipidaemia (PPARα). It is therefore of interest that 11βHSD1 is down-regulated by PPARα (Hermanowski-Vosatka *et al.* 2000) and PPARγ (Berger *et al.* 2001; Laplante *et al.* 2003), whilst 11βHSD2 is suppressed by PPARδ (Julan *et al.* 2005).

11βHSDs are also altered in important pathophysiological conditions which are themselves risk factors for cardiovascular disease. Obesity is associated with an increase in adipose 11βHSD1 expression and activity (Livingstone *et al.* 2000a; Masuzaki *et al.* 2001; Rask *et al.* 2001; Rask *et al.* 2002; Lindsay *et al.* 2003; Wake *et al.* 2003;Westerbacka *et al.* 2003) and a reduction in hepatic 11βHSD1 (Rask *et al.* 2002; Rask *et al.* 2001; Stewart *et al.* 1999). By contrast, in what is thought to represent a protective metabolic adaptation to caloric excess, a high fat diet results in

rapid down-regulation of adipose 11βHSD1 (Morton *et al.* 2004b). Furthermore, given the severe hypertensive phenotype in 11βHSD2 deficiency states, it is interesting, but perhaps unsurprising, to find that polymorphisms of 11βHSD2 influence renal sodium handling and may, therefore, contribute to the pathogenesis of essential hypertension (Lovati *et al.* 1999; Williams *et al.* 2005).

The mechanisms by which humoral factors and disease states regulate 11βHSD remain largely unclear. However, intriguing new data have found that methylation of the 11βHSD2 gene is associated with a decrease in its expression (Alikhani-Koopaei *et al.* 2004). As DNA methylation of many genes changes with age, disease states, and environmental signals including diet, this epigenetic mechanism may provide one explanation for the pathological changes in 11βHSD expression which are evident under these conditions.

1.2.7 Regulation of 11β-hydroxysteroid dehydrogenases by inflammatory mediators

Inflammatory cytokines are perhaps the most extensively studied group of mediators which regulate 11βHSDs (see Table 1.1). Their effects on 11βHSD activity are of great interest and relevance considering that atherosclerosis and the predisposing risk factors, obesity and the metabolic syndrome, are now widely recognised to be inflammatory conditions (Ross 1999; Libby *et al.* 2002; Lee & Pratley 2005). The majority of studies to date have reported changes in 11βHSDs which favour an increase in glucocorticoid availability during inflammation. TNFα and/or IL-1β increase 11βHSD1 activity and/or expression in human aortic smooth muscle cells (SMCs) (Cai *et al.* 2001), rat glomerular mesangial cells (Escher *et al.* 1997), human adipocytes (Tomlinson *et al.* 2001; Handoko *et al.* 2000; Friedberg *et al.* 2003), human osteoblasts (Cooper *et al.* 2001), and human ovarian epithelial cells (Yong *et al.* 2002). In both osteoblasts (Cooper *et al.* 2001) and human aortic SMCs (Cai *et al.* 2001), an accompanying downregulation in 11βHSD2 is also observed. These coordinated changes would be expected to increase local availability of active glucocorticoids and contribute to feedback regulation of inflammation.

However, the effects of cytokines on the 11βHSDs are not entirely consistent, as TNFα has no effect on 11β-reductase activity in cultured human hepatocytes (Tomlinson *et al.* 2001). Furthermore, in circulating monocytes, 11βHSD1 expression is not up-regulated by the pro-inflammatory cytokines TNFα or IL-1β but is induced during differentiation into macrophages, and also following exposure to the T-helper (Th2) lymphocyte-derived cytokines, IL-4 and IL-13 (Thieringer *et al.* 2001). Additionally, whilst IL-1β modestly increases 11βHSD1 expression (but not activity) in cultured human amnion fibroblasts, no such effect is evident following stimulation with TNFα, although both TNFα and IL-1β exert a synergistic effect on the up-regulation of 11βHSD1 by dexamethasone (Sun & Myatt 2003).

Finally, recent studies have highlighted the potential importance of the state of cellular proliferation and/or differentiation in modulating the regulation of 11βHSDs by cytokines. Basal 11βHSD1 expression is lower, and 11βHSD2 expression higher, in human cell lines derived from ovarian adenocarcinomas compared with human ovarian surface epithelial (HOSE) cells obtained by primary culture (Gubbay *et al.* 2005). Moreover, treatment with the inflammatory cytokine IL-1α selectively enhances 11βHSD1 in HOSE cells but not in carcinoma cells, whilst 11βHSD2 expression is up-regulated in some carcinoma cell lines but not in HOSE cells. Hence, changes in the regulation of 11βHSD may accompany, or even determine, changes in cellular differentiation or proliferation (Rabbitt *et al.* 2003). Bearing this last point in mind, it should be noted that all studies so far which have investigated the regulation of 11βHSDs by inflammatory mediators have utilised cell culture systems, which undoubtedly alter the natural cell phenotype.

Table 1.1 Regulation of 11βHSDs by inflammatory mediators

Study	Cell type	Cytokine	Dose	11βHSD1		11βHSD2	
				Activity	mRNA	Activity	mRNA
Escher *et al.*, 1997	Glomerular mesangial cells	TNFα	10nM	↑	↑	None	-
	"	IL-1β	10nM	↑	↑	None	-
Tetsuka *et al.*, 1999	Granulosa cells	IL-1β	50ng/ml	-	↑	-	-
Handoko *et al.*, 2000	Adipose stromal cells	TNFα	0.1-10 ng/ml	↑	↑	-	-
Sun and Myatt, 2001	Amnion fibroblasts	TNFα	10ng/ml	↔	-	-	-
	"	IL-1β	10ng/ml	↑	-	-	-
Tomlinson *et al.*, 2001	Adipose stromal cells	TNFα	10ng/ml	↑	-	-	-
	"	IL-1β	10ng/ml	↑	-	-	-
	"	IL-6	1-10ng/ml	↑/↔	-	-	-
	Hepatocytes	TNFα	10ng/ml	↔	-	-	-
Cai *et al.*, 2001	Aortic smooth muscle cells	TNFα	100-200ng/ml	↑	↑	↔	↓
	"	IL-1β	10-20ng/ml	↑	↑	↔	↓
Cooper *et al.*, 2001	Osteoblasts	TNFα	10ng/ml	↑	↑	↓	↓
	"	IL-1β	10ng/ml	↑	↑	↓	↓
Thieringer *et al.*, 2001	Monocytes / macrophages	TNFα	10ng/ml	↔	↔	None	-
	"	IL-1β	10ng/ml	↔	↔	None	-
	"	IL-4, IL-13	50ng/ml	↑	-	None	-
	"	LPS	3ng/ml	-	↑	None	-
Yong *et al.*, 2002	Ovarian surface epithelial cells	IL-1α	0.5ng/ml	↑	↑	-	-
	"	IL-1β	0.5ng/ml	↑	↑	-	-
Friedberg *et al.*, 2003	Adipocytes	TNFα	0.6nM	↑	-	-	-
	"	IL-1β	0.6nM	↑	-	-	-
Rae *et al.*, 2004	Ovarian surface epithelial cells	IL-1α	0.5ng/ml	-	↑	-	-
Gubbay *et al.*, 2005	Ovarian ca. / epithelial cells	IL-1α	0.5ng/ml	-	↔/↑	-	↑/↔

1.3 Intra-vascular glucocorticoids

The focus of this thesis is the regulation of vascular 11βHSDs and, hence, local glucocorticoid action, by inflammatory mediators. There is increasing evidence that, as well as the systemic effects of glucocorticoids on the cardiovascular system (discussed in Section 1.1.3.3), direct interaction of these steroids with cells of the vessel wall may contribute to their associations with cardiovascular disease. As inflammation also has direct effects on vascular function, the potential link between these interactions is of great interest.

The previous section has described how local glucocorticoid action is determined by tissue-specific pre-receptor metabolism by the 11β-hydroxysteroid dehydrogenases. Both isozymes of 11βHSD are present in the vessel wall and their roles in the regulation of the direct vascular effects of glucocorticoids are discussed in detail in the following section.

1.3.1 Vascular glucocorticoid action

The presence of both mineralocorticoid and glucocorticoid receptors within cells of the vascular wall (Ullian 1999; Christy *et al.* 2003) suggests that glucocorticoids can interact directly with the vasculature to influence aspects of vascular function and structure. This interaction may be site-specific as there is evidence that the cellular distribution of these receptors is territory-dependent. For example, MR is present in the endothelial and smooth muscle cells of rabbit aorta and pulmonary artery but not in the arterioles and capillaries (Lombes *et al.* 1992), whilst in the rat mesenteric microcirculation, both MR and GR are more abundant in the wall of the arterioles and venules, as compared with the capillaries (DeLano & Schmid-Schonbein 2004). Regulation of 11βHSD activity within the vessel wall, for example by inflammatory mediators, may play an important role in modulating direct glucocorticoid-mediated changes in vascular function and structure.

1.3.2 Vascular 11β-hydroxysteroid dehydrogenases

Both 11βHSD1 and 11βHSD2 are present in the vessel wall (Hadoke *et al.* 2001; Christy *et al.* 2003; Brem *et al.* 1998) (Figure 1.5). The cellular localisation of each isozyme is debated, and may be both species and site-specific. Vascular endothelial cells isolated from rats appear to express both 11βHSD1 and 11βHSD2 (Brem *et al.* 1998), although studies from this department have identified only the 11βHSD2 isoform in the endothelium of both rat and mouse aorta (Christy *et al.* 2003). The expression of 11βHSD isozymes in vascular smooth muscle is also controversial.

Studies in the rat and mouse have suggested that vascular SMCs express only 11βHSD1 (Brem *et al.* 1998; Christy *et al.* 2003), whereas both isozymes have been identified in human aortic and coronary artery smooth muscle cells (Cai *et al.* 2001; Hatakeyama *et al.* 1999). Whilst these differences may be attributable to species and site specific differences in 11βHSD expression, direct comparison between studies is problematic due to the variety of investigative techniques employed (eg. intact vessels vs cultured SMCs). There is data to suggest that there are territory-dependent differences in 11βHSD activity in the rat at least, as higher 11βHSD activity is present in mesenteric arteries compared with aorta (Walker *et al.* 1991).

As discussed previously in Section 1.2.5, 11βHSD1 is generally accepted to act predominantly as a reductase *in vivo*, catalysing regeneration of active glucocorticoids. However, there is some evidence that 11βHSD1 has bi-directional capability in vascular tissue as significant 11β-dehydrogenase activity has been detected in endothelium-denuded aorta and smooth muscle cells apparently devoid of 11βHSD2 expression (Brem *et al.* 1995). Furthermore, 11βHSD1 antisense oligonucleotides diminish conversion of glucocorticoids to their inert 11-keto metabolites in rat aortic rings (Souness *et al.* 2002) suggesting the presence of 11βHSD1 dehydrogenase activity. Attempts to clarify the directionality of each isozyme within vascular tissue using conventional activity assays in homogenised tissue preparations (Christy 2003) have been limited by a lack of co-factor specificity between isozymes of 11βHSD in mice (Walker *et al.* 1992b).

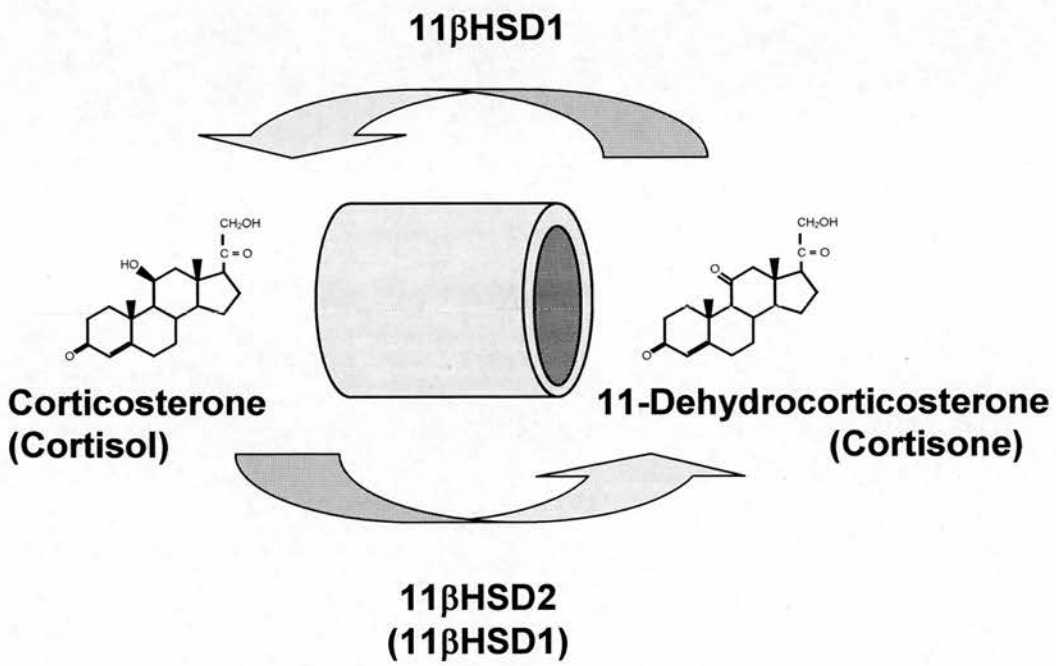

Figure 1.5 Intra-vascular glucocorticoid metabolism by the 11βHSDs

Interconversion of active glucocorticoids (corticosterone in rodents, cortisol in man) with their inert 11-keto metabolites (11-dehydrocorticosterone and cortisone, respectively) occurs in vascular tissue through the actions of the type 1 and type 2 isozymes of 11β-hydroxysteroid dehydrogenase.

However, there is clearly a need to resolve the relative contributions of these isozymes to 11β-reductase and 11β-dehydrogenase activities within vascular tissue to understand the regulation of the 11βHSDs.

1.3.3 Intra-vascular glucocorticoid effects

1.3.3.1 Effects of glucocorticoids on vascular morphology

Angiogenesis, the formation of new blood vessels from the existing vascular network, is a complex process which contributes to both physiological and pathophysiological processes. Tightly regulated angiogenesis is essential for embryonic development and during the reproductive cycle (Folkman 2001), whilst uncontrolled angiogenesis is a component of disorders including neoplasia and proliferative diabetic retinopathy, and can be stimulated by inflammation (Conway *et al.* 2001).

Pharmacological doses of glucocorticoids inhibit angiogenesis both *in vitro* and *in vivo* (Folkman *et al.* 1983; Maragoudakis *et al.* 1989; Nicosia & Ottinetti 1990; Folkman & Ingber 1987; Hori *et al.* 1996), and they therefore have therapeutic potential (Kinnaird *et al.* 2003; Siemann *et al.* 2004). However, once again, the adverse effects of systemic glucocorticoid therapy have restricted their use in this regard. Recent studies within this department have demonstrated that mice with genetic inactivation of 11βHSD1 have enhanced angiogenesis *in vitro* and *in vivo* within implanted sponges, wounds and infarcted myocardium (Small *et al.* 2005). These exciting new data suggest that endogenous glucocorticoids, including those generated locally by 11βHSD1, exert tonic inhibition of angiogenesis. Thus, inhibition of 11βHSD1 may provide a therapeutic approach to improve healing of ischaemic or injured tissues.

In addition to their potent angiostatic properties, glucocorticoids also influence a number of critical processes involved in the vascular response to injury.

Glucocorticoids are known to inhibit vascular smooth muscle cell growth (Berk *et al.* 1988; Longenecker *et al.* 1982). However, they may also paradoxically promote cell proliferation, as they attenuate the activity of nitric oxide (Walker *et al.* 1995b; Mangos *et al.* 2000), a potent inhibitor of cell growth, and enhance production of endothelin-1 (Morin *et al.* 1998) and angiotensin II (Fishel *et al.* 1995), which both stimulate cell growth (Berk *et al.* 1989; Griffin *et al.* 1991). Thus there may be complex interactions between glucocorticoids and the vasculature to regulate vascular remodelling.

An important clinical consequence of the vascular response to injury is the process of neointimal proliferation. Neointimal lesions develop in response to injury and/or inflammation (for example in atherosclerosis or following metal stent deployment during revascularisation) as a result of migration and proliferation of smooth muscle cells on the luminal surface of the vessel wall (Wainwright *et al.* 2001). Glucocorticoids, administered both locally and systemically in animal models, have been shown to inhibit neointimal lesion development (Macdonald L, unpublished observations; Villa *et al.* 1994; Guzman *et al.* 1996; Van Put *et al.* 1995; Petrik *et al.* 1998; Strecker *et al.* 1998; Pires *et al.* 2005). However, there is some evidence that this inhibition of neointimal formation occurs in conjunction with adverse vascular morphological changes pointing to a loss of vascular integrity (Pires *et al.* 2005). Additionally, the inhibition of lesion development by glucocorticoids was not universal (Karim *et al.* 1997; Lincoff *et al.* 1997) and, whilst one clinical study suggested that systemic prednisolone reduced the incidence of in-stent restenosis (Versaci *et al.* 2002), most clinical trials have been disappointing (Pepine *et al.* 1990; Reimers *et al.* 1998; Rab *et al.* 1991). The potential for the 11βHSDs to modulate these morphological changes, through their effects on local vascular glucocorticoid availability, is currently under investigation in this department. A recently published study has demonstrated that systemic administration of a selective inhibitor of 11βHSD1 dramatically slows plaque progression in a murine model of atherosclerosis (Hermanowski-Vosatka *et al.* 2005). It is likely that the influence of glucocorticoids on neointimal lesion development and atherosclerosis is mediated, at

least in part, by their anti-inflammatory properties, since inhibition of inflammation also reduces neointimal proliferation (Miller *et al.* 2001).

1.3.3.2 Effects of glucocorticoids on inflammation

A critical component of the pathophysiology of vascular diseases such as atherosclerosis or vessel injury is the inflammatory response to endothelial cell injury (Ross 1999; Ross 1993; Ross 1986). In the initial stages of atheroma formation, endothelial cell activation leads to expression of adhesion molecules, such as P-selectin (Johnson *et al.* 1997) and vascular cell adhesion molecule-1 (Li *et al.* 1993), and increased vascular permeability. In response to chemokines such as macrophage chemoattractant protein-1, leukocytes adhere to the endothelium and migrate into the sub-endothelial space (Gu *et al.* 1998). These leukocytes then become activated and release inflammatory mediators such as interleukin-1 and tumour necrosis factor α, causing further recruitment and activation of inflammatory cells and stimulation of endothelial and vascular smooth muscle cells to take part in the inflammatory response. Vascular SMC proliferation, driven by growth factors such as platelet-derived growth factor (Ferns *et al.* 1991) and basic fibroblast growth factor (Lindner & Reidy 1991), subsequently results in neointimal proliferation and lesion formation. As described in Section 1.3.3.1, acute vessel injury can also result in an excessive inflammatory response and subsequent neointimal proliferation.

Glucocorticoids exert an array of anti-inflammatory and anti-proliferative actions, which have been discussed previously in Section 1.1.3.2. Many of these effects are likely to be mediated by direct local interaction of glucocorticoids with blood vessels, inflammatory cells within the vasculature and mediators of the inflammatory response (Barnes & Adcock 1993). Glucocorticoids alter the recruitment of neutrophils and macrophages to the site of vessel injury (Poon *et al.* 2001; Asai *et al.* 1993; Naito *et al.* 1992) by decreasing expression of cytokines, chemokines and adhesion molecules (Poon *et al.* 1996). Glucocorticoids also inhibit leukocyte activation and proliferation, promote phagocytosis of apoptotic neutrophils and inhibit T cell synthesis and induce lymphocyte apoptosis. In a recent clinical trial,

local vascular glucocorticoid therapy (administered via dexamethasone-eluting stents) reduced systemic markers of inflammation, such as C-reactive protein, and improved outcomes (Patti *et al.* 2005). As discussed in Section 1.3.3.1, it is likely that the influence of glucocorticoids on the vascular response to injury is in part explained by their immunomodulatory effects.

Whilst pharmacological doses of glucocorticoids are undoubtedly powerful immunosupressants, the immunomodulatory effects of physiological glucocorticoids in the vascular inflammatory process is less clear. Endogenous glucocorticoids are thought to serve as a "brake" to the immune response, protecting tissues from the adverse effects of an excessive inflammatory response (Munck *et al.* 1984), and are an absolute requirement to survive endotoxin or cytokine challenge (Bertini *et al.* 1988). Furthermore, the activation of the HPA axis by numerous pro-inflammatory cytokines (Turnbull & Rivier 1995; Turnbull *et al.* 2003) suggests that feedback regulation of inflammation acts to limit an over-vigorous immune response. Thus, the regulation of local vascular glucocorticoid levels by the 11βHSDs, and the influence of inflammation on this process is a pertinent topic. As discussed in Section 1.2.7, there is evidence of regulation of 11βHSDs by inflammatory cytokines in cultured vascular SMCs (Cai *et al.* 2001). If this process also occurs in intact arteries, there may also be a process of feedback regulation of inflammation within the vessel wall itself.

1.3.3.3 Effects of glucocorticoids on vascular tone

The local regulation of vascular tone involves complex interactions between the cells of the endothelium and vascular smooth muscle. The endothelium, in response to stimulation (by factors such as acetylcholine, bradykinin, shear stress etc) produces a wide number of vasoactive substances, including vasodilators such as nitric oxide, prostaglandins and endothelium-derived hyperpolarising factor (EDHF), and vasoconstrictors such as endothelin-1, angiotensin II and thromboxanes (Figure 1.6). Nitric oxide, a key mediator of vascular tone, is synthesised from L-arginine by a triad of isozymes, the nitric oxide synthases (NOS) (Figure 1.7).

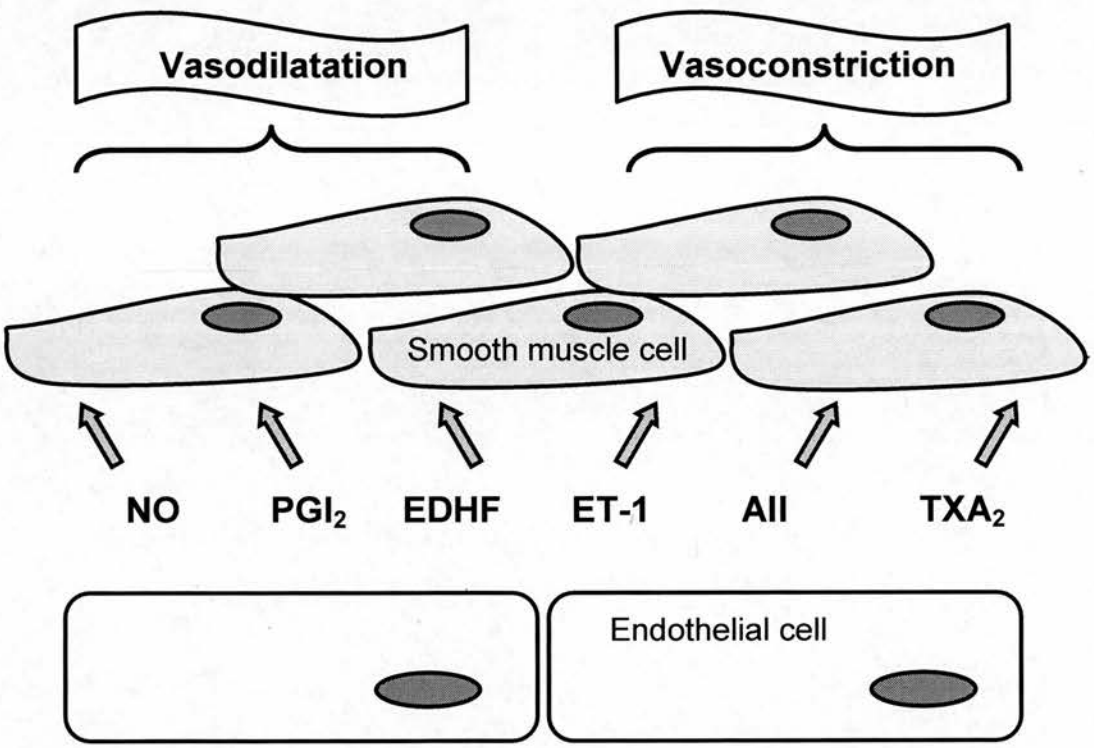

Figure 1.6 Influence of the endothelium on vascular tone

The endothelium, in response to stimulation, releases a variety of vasodilators and vasoconstrictors. NO = nitric oxide; PGI_2 = Prostacyclin; EDHF = endothelium-derived hyperpolarising factor; ET-1 = endothelin-1; AII = angiotensin II, TXA_2 = thromboxane A_2.

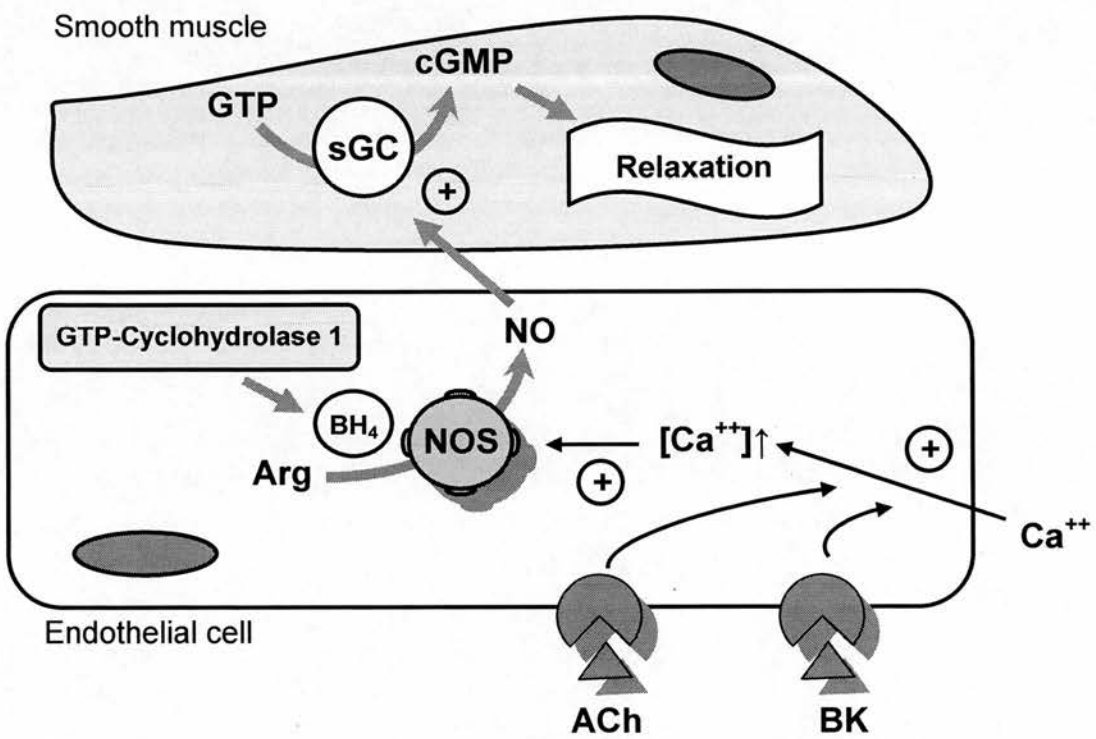

Figure 1.7 Nitric oxide mediated vasodilatation

Upon stimulation of the endothelium by agents such as bradykinin (BK) or acetylcholine (ACh), nitric oxide is synthesized by constitutive (endothelial and neuronal) and inducible isoforms of nitric oxide synthase (eNOS, nNOS and iNOS, respectively). The production of the essential cofactor for NOS, tetrahydrobiopterin, BH_4, is regulated by the activity of GTP cyclohydrolase-1. Nitric oxide acts on smooth muscle cells to increase generation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) by soluble guanylyl cyclase (sGC), resulting in vasorelaxation.

The NOS isozymes require an essential cofactor, tehtrahydrobiopterin (BH_4), whose availability depends on the activity of the rate-limiting enzyme guanosine triphosphate (GTP) cyclohydrolase I.

Unlike the constitutively expressed endothelial (eNOS) and neuronal NOS (nNOS) isoforms, inducible nitric oxide synthase (iNOS) is activated in response to inflammatory mediators and is capable of sustained production of large quantities of nitric oxide (Moncada *et al.* 1991). Inflammation is associated with impaired endothelium-dependent vasomotion (Chia *et al.* 2003b; Kofler *et al.* 2005), and excessive generation of nitric oxide by iNOS may account for the vascular dysfunction evident in a number of inflammatory conditions including sepsis and endotoxaemia (Hallemeesch *et al.* 2003; Chauhan *et al.* 2003).Glucocorticoids have been shown to inhibit iNOS (Korhonen *et al.* 2002) and GTP cyclohydrolase (Mitchell *et al.* 2004) and improve sepsis-induced vascular dysfunction (Mansart *et al.* 2003).

It is well recognised that glucocorticoids contribute to the maintenance of vascular tone *in vivo*. As discussed in Section 1.1.3.3, glucocorticoid excess is associated with hypertension, whilst deficiency results in hypotension. Although some of the influences of glucocorticoids on systemic blood pressure are mediated through effects on cardiac output and renal salt and water homeostasis, there is increasing evidence that glucocorticoids interact directly with the vessel wall to influence contractility. Glucocorticoids potentiate pressor-mediated vasoconstriction (Ullian 1999; Walker & Williams 1992) by modulating vascular smooth muscle cell signalling mechanisms (Sato *et al.* 1994; Sato *et al.* 1992; Yasunari *et al.* 1990). Furthermore, acetylcholine-mediated vasodilatation is attenuated by glucocorticoids (Walker *et al.* 1995b; Mangos *et al.* 2000), which also reduce the activity of other vasodilators (eg prostaglandins, nitric oxide) (Rosenbaum *et al.* 1986; Kelly *et al.* 1998; Wallerath *et al.* 1999; Simmons *et al.* 1996). It is likely that the mechanism of impaired cholinergic dilatation following glucocorticoid therapy involves abnormalities of the endothelial nitric oxide system (Hadoke *et al.* 2001; Mangos *et al.* 2000). Despite the evidence that glucocorticoids directly influence mechanisms

involved in the regulation of vascular tone, there are some important negative findings which deserve attention. Although longer term treatment of healthy volunteers with systemic glucocorticoids results in an increase in blood pressure and impaired acetylcholine-mediated NO release (Mangos *et al.* 2000), short term systemic or intra-arterial infusion of cortisol does not appear to influence physiological or biochemical markers of nitric oxide activity (Williamson *et al.* 2005; Mangos *et al.* 2000), supporting the concept that glucocorticoid-mediated endothelial cell dysfunction may be secondary to effects of elevated blood pressure.

Early observations that either congenital deficiency or pharmacological inhibition (by liquorice) of 11βHSD2 (Ulick *et al.* 1979; Stewart *et al.* 1988; Epstein *et al.* 1977) was associated with hypertension raised the possibility that 11βHSD activity played an important role in the regulation of vascular tone. Whilst the hypertension related to 11βHSD2 deficiency was initially attributed to sodium retention as a result of MR activation by glucocorticoids in the distal nephron (Stewart *et al.* 1988), there is increasing evidence that the activity of 11βHSDs within the vessel wall directly influence vascular tone. This concept is supported by findings that 11βHSD activity is altered in vessels from hypertensive rats (Takeda *et al.* 1994a; Takeda *et al.* 1994b; Takeda *et al.* 1993). The relevance of the 11βHSDs in modulating the effects of glucocorticoids on vascular tone have been explored in more detail through a series of studies using pharmacological inhibition or genetic inactivation of 11βHSD. However, the precise role for each isozyme in the regulation of vascular tone remains incompletely understood.

In the rat, systemic inhibition of 11βHSD by liquorice derivatives results in hypertension which is associated with endothelial dysfunction (Quaschning *et al.* 2001; Ruschitzka *et al.* 2001). In man, pharmacological inhibition of 11βHSD with glycyrrhetinic acid enhances cortisol-mediated dermal vasoconstriction (Teelucksingh *et al.* 1990). More recently, *in vitro* studies have demonstrated that exposure to pharmacological inhibitors of 11βHSD such as glycyrrhetinic acid (Walker *et al.* 1992c), carbenoxolone (Brem *et al.* 1997) or chenodeoxycholic acid (a bile acid which is an endogenous inhibitor of 11βHSD) (Morris *et al.* 2004) enhances

glucocorticoid-potentiated vasoconstriction in isolated vessels, suggesting that these effects may be due to direct changes in glucocorticoid availability within the vessel wall itself. However, the lack of selectivity of inhibitors of 11βHSD has restricted the conclusions that can be drawn from these studies. Additionally, direct adverse effects of these pharmacological agents on vascular function have been noted (Walker *et al.* 1994a; Ullian *et al.* 1996), and thus the literature must be interpreted with caution. Nevertheless, considering that these inhibitors consistently potentiate the vascular effects of glucocorticoids, it is likely that their effects are mediated by inhibition of inactivation rather than reactivation of steroids within the vessel wall.

Studies using mice with genetic inactivation of 11βHSD1 (Kotelevtsev *et al.* 1997; Hadoke *et al.* 2001) or 11βHSD2 (Kotelevtsev *et al.* 1999; Hadoke *et al.* 2001) support the conclusions that have been drawn from pharmacological studies. The intra-vascular regeneration of glucocorticoids does not appear to be important for the maintenance of vascular tone, as deficiency of 11βHSD1 has no effect on aortic function or blood pressure (Kotelevtsev *et al.* 1997; Hadoke *et al.* 2001). By contrast, mice with genetic inactivation of 11βHSD2 have a hypertensive phenotype (Kotelevtsev *et al.* 1999), and exhibit enhanced vascular contractility as a result of impaired endothelium-derived nitric oxide production (Hadoke *et al.* 2001). Furthermore, incubation of rat aortic rings with 11βHSD2 antisense oligonucleotides enhances glucocorticoid-potentiated vasoconstriction (Souness *et al.* 2002).

These findings have led to the proposal that 11βHSD2 plays a key role in vessels by protecting the endothelium from the adverse effects of excessive glucocorticoid exposure. However, attributing the endothelial cell dysfunction evident in 11βHSD2 deficiency/inhibition solely to direct effects within the vessel wall is likely to be an over-simplification. Recent studies using mouse aortic rings have failed to show an effect of glucocorticoids on endothelial cell function, even in vessels from mice with genetic inactivation of 11βHSD2 (Christy *et al.* 2003), suggesting that indirect mechanisms (relating to sodium retention and/or hypertension) contribute to the abnormal vascular phenotype which is evident. Nonetheless, the mechanisms of the hypertension related to 11βHSD2 deficiency/inhibition are beginning to be

unravelled: pharmacological inhibition of 11βHSD2 lowers eNOS protein and activity levels and enhances levels of the vasopressor endothelin 1 in rat aorta; a phenomenon ameliorated by either aldosterone receptor (Quaschning *et al.* 2001) or endothelin receptor A antagonism (Ruschitzka *et al.* 2001). These data suggest that there is undoubtedly a role for the 11βHSDs in modulating the effects of glucocorticoids on vascular tone, although the precise mechanisms remain elusive.

1.3.3.4 Effects of glucocorticoids on endogenous fibrinolysis

Endogenous fibrinolysis is a complex process whereby clot, produced by the action of coagulation factors, is degraded by the hydrolytic cleavage of fibrin by plasmin. Plasmin, a serine protease, is produced from its precursor plasminogen through the co-ordinated action of a number of enzymes and inhibitors. An adequate fibrinolytic response is essential to maintain arterial patency and to avoid intravascular fibrin formation, vessel occlusion and tissue infarction. The fibrinolytic system, apart from its role in maintaining blood flow, is also involved in macrophage migration (Saksela & Rifkin 1988), tumour invasion (Dano *et al.* 1985), embryogenesis (Saksela & Holthofer 1987) and ovulation (Strickland & Beers 1976).

Initiation of intravascular fibrinolysis (Figure 1.8) depends on the conversion of plasminogen, produced in the liver (Raum *et al.* 1980), to plasmin through cleavage of the Arg^{561}-Val^{562} peptide bond by tissue plasminogen activator (t-PA) (Astedt 1979) (Kok 1979). The principle site of t-PA synthesis, storage and release is the endothelium (Levin & del Zoppo 1994). There is a dynamic intracellular storage pool of t-PA (Eijnden-Schrauwen *et al.* 1995) which is released in response to stimulation by many physiological and pharmacological factors including cytokines, vasodilators and environmental factors (smoking, alcohol) (Emeis 1992), without the need for *de novo* protein synthesis (Tranquille & Emeis 1989). t-PA activity exhibits circadian variation, with lowest activity in the early morning (as a consequence of high plasminogen activator inhibitor-1 (PAI-1) concentrations) (Andreotti & Kluft 1991). There is rapid hepatic clearance of t-PA, such that the plasma half life is only a few minutes (Chandler *et al.* 1997).

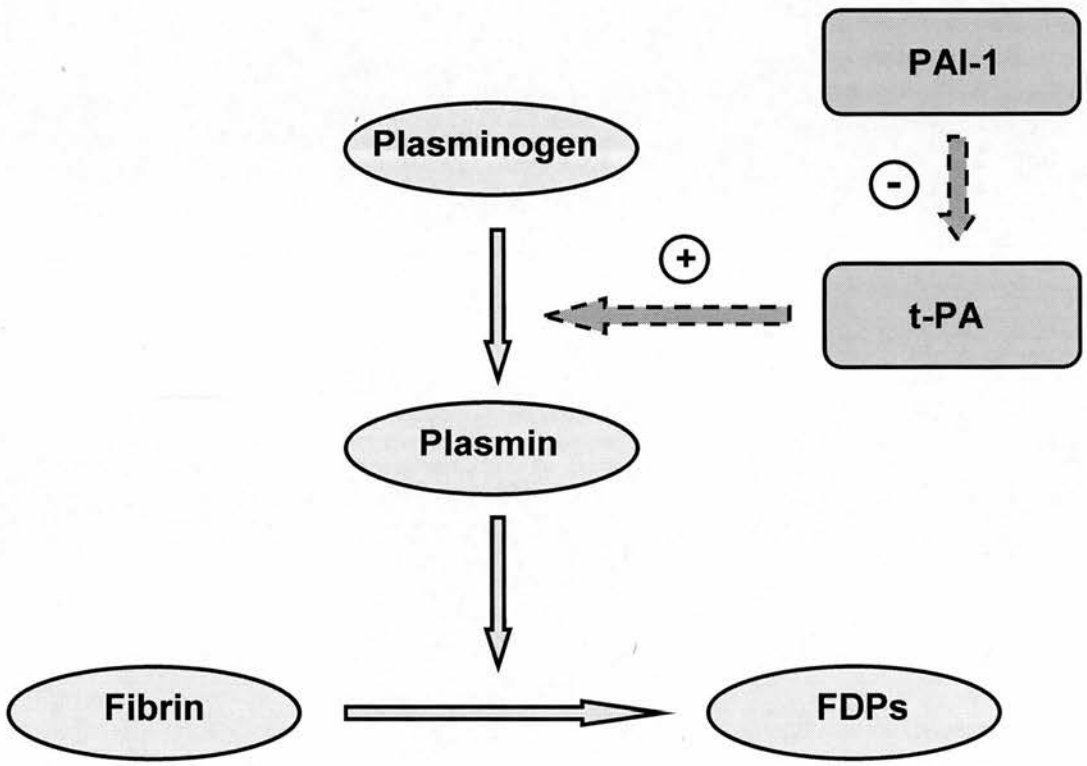

Figure 1.8 The roles of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (PAI-1) in intra-vascular fibrinolysis

During intra-vascular fibrinolysis, fibrin clot is degraded by the enzyme plasmin into fibrin degradation products (FDPs). Tissue plasminogen activator (t-PA) is released by the endothelium to activate plasmin from plasminogen. The activity of t-PA is inhibited by the protease inhibitor plasminogen activator inhibitor-1 (PAI-1).

The action of t-PA is inhibited when it is bound to plasminogen activator inhibitor type 1 (PAI-1). PAI-1 is produced predominantly by the endothelium and vascular smooth muscle, but can also be synthesised from liver and platelets (Sprengers *et al.* 1986) (Simpson *et al.* 1991). Elevated PAI-1 levels and impaired t-PA activity are associated with increased cardiovascular risk (Kohler & Grant 2000; Juhan-Vague *et al.* 1999), so this dynamic aspect of endothelial cell function and fibrinolytic balance may be directly relevant to the pathogenesis of atherothrombosis.

In vivo effects of glucocorticoids on fibrinolytic function have not been clearly elucidated. A recent study found no effect on circulating PAI-1 levels in healthy volunteers treated with dexamethasone for 5 days (Brotman *et al.* 2005). However, patients with glucocorticoid excess, either endogenous or exogenous, have elevated circulating PAI-1 levels and a hyper-coagulable state (Sartori *et al.* 2000; Fatti *et al.* 2000; Patrassi *et al.* 1985; Ikkala *et al.* 1985; Patrassi *et al.* 1992; Sartori *et al.* 1999). Studies *in vitro* suggest that PAI-1 release is increased by glucocorticoids (Udden *et al.* 2002; Fukumoto *et al.* 1992; Reinders *et al.* 1992; Halleux *et al.* 1999; Morange *et al.* 1999), and there is also evidence that dexamethasone can augment cytokine stimulated PAI-1 release (Yamamoto *et al.* 2004; He *et al.* 2000). Animal models also suggest that *in vivo* glucocorticoid therapy increases PAI-1 levels, and decrease t-PA release (van Giezen & Jansen 1992; van Giezen *et al.* 1994), a finding mirrored in studies examining PAI-1 and t-PA expression in cultured hepatocytes (Uno *et al.* 1998). Whilst there is some suggestion that glucocorticoids may actually increase t-PA release (Gelehrter *et al.* 1987), this is in conjunction with an even greater increase in PAI-1, supporting the concept that glucocorticoids influence fibrinolysis to favour a prothrombotic state.

1.4 Hypothesis and aims

There is good evidence that glucocorticoids directly influence many aspects of vascular function, and that these effects are modulated, at least in part, by the isozymes of 11βHSD. Inflammation is a key component of many vascular disease processes. The finding that inflammatory mediators influence local availability of glucocorticoids in cultured cells through regulation of the 11βHSDs suggests that inflammation may influence local vascular glucocorticoid action.

1.4.1 Hypothesis

It was hypothesised that both 11βHSD1 and 11βHSD2 activities are present in the intact vessel, where they act, as exclusive reductase and dehydrogenase respectively, to regulate local availability of glucocorticoids. Further, it was proposed that inflammatory cytokines selectively up-regulate 11βHSD1 in intact vascular tissue to enhance the local availability of active glucocorticoids, providing local feedback regulation of inflammation. Finally, in addition to the adverse consequences of enhanced glucocorticoid availability on endothelial cell vasomotor function, it is proposed that there is an impairment of fibrinolysis, promoting a prothrombotic state.

1.4.2 Aims

1. To determine the presence, and specific directionalities, of 11βHSD1 and 11βHSD2 in the vessel wall *in vitro* and *in vivo*.

2. To investigate the influence of inflammatory mediators on 11βHSD activity in intact vascular tissue *in vitro* and *in vivo*.

3. To assess the impact of variations in glucocorticoid availability on endothelial cell vasomotor and fibrinolytic function *in vivo*.

Chapter Two

Materials and Methods

2.1 Materials

Unless otherwise stated, all chemicals and reagents were purchased from Sigma, UK. All radioactivity was purchased from Amersham, UK. HPLC grade solvents were purchased from Rathburn Chemicals, UK. Drugs for clinical studies were purchased from Merck Biosciences AG, Switzerland.

2.1.1 Buffers and solutions

C buffer: 63 g glycerol, 8.77 g NaCl, 186 mg EDTA and 3.03 g Tris made up to 500 ml with distilled water, pH 7.7, stored at 4°C.

Evans Blue: 25 mg Evans blue, in 5 ml distilled water (0.5% w/v).

Hanks' Balanced Salt Solution: 1.26 mM $CaCl_2$, 0.49 mM $MgCl_2.6H_2O$, 0.41 mM $MgSO_4.7H_2O$, 5.3 mM KCl, 0.4 mM KH_2PO4, 137.9 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO4, 5.56 mM D-glucose, 0.03 mM Phenol Red.

Homogensiation buffer: 100 g glycerol, 300 mg Tris and 186 mg EDTA made up to 500 ml in distilled water, pH 7.5. Stored at 4°C and supplemented with 7.7 mg/50ml dithiothreitol (DTT) immediately prior to use.

Krebs-Ringer buffer (KRB): NaCl 118 mM, KCl 3.8 mM, KH_2PO_4 1.19 mM , $CaCl_2$ 2.54 mM, $MgSO_4$ 1.19 mM, $NaHCO_3$ 25 mM in distilled water, pH 7.4.

Modified Krebs'-Henseleit Buffer (KHB): NaCl 118.3 mM, KCL 4.7 mM, $MgSO_4.7H_2O$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, EDTA 0.026 mM, $CaCl_2.2H_2O$ 1.8 mM, Glucose 11.1 mMin distilled water, pH 7.4. Stored at 4°C and supplemented with 2% bovine serum albumin immediately before use.

Nicotinamide Adenine Dinucleotide (NAD): 17.1 mg in 1 ml C buffer (final concentration 25 mM).

Nicotinamide Adenine Dinucleotide Phosphate (NADP): 7.65 mg in 1 ml KRB buffer (final concentration 10 mM).

Phosphate buffered Saline (PBS): 0.1 M phosphate buffer with 137 mM NaCl, 2.7 mM KCl in distilled water, pH 7.4, autoclaved before use.

Placental Homogenate: 2 Wistar rat placentas mechanically homogenised in 1ml homogenisation buffer and stored in 300 µl aliquots at -80°C.

2.1.2 Drugs and radiolabelled steroids used in animal studies

Acetylcholine (ACh, chloride salt, FW 181.7): 54.51 mg ACh dissolved in 30 ml distilled water (final concentration 10^{-2} M). 500 µl aliquots stored at -20°C.

Bradykinin (BK, triacetate salt, FW 1240.36): 10 mg BK dissolved in 8.06 ml distilled water (final concentration 10^{-3} M). 450 µl aliquots stored at -20°C.

[^{3}H]-Corticosterone: Commercial stock solutions of [^{3}H]-Corticosterone in ethanol (with concentrations of 13.7 nmol/ml) were stored at -20°C.

Corticosterone: 6.93 mg corticosterone was made up in 1 ml of methanol (final concentration 20 mM) and stored at -20°C.

11-Dehydrocorticosterone: 10 mg 11-dehydrocorticosterone was made up to 10 mls ethanol (2.9 mM concentration) and this was further diluted ten-fold in ethanol to give a 290 µM solution and again to give a 2.9 µM solution. These stock solutions were stored at -20°C.

[^{3}H]-11-Dehydrocorticosterone: [^{3}H]-11-Dehydrocorticosterone was synthesised in-house (see Section 2.6), resuspended in ethanol (to give concentrations of approximately 10-15 nmol/ml) and stored at -20°C.

Etanercept: 500 µl of a 25 mg/ml solution of etanercept (Enbrel® Amgen Inc, USA) was added to 12 mls distilled water (final concentration 1 mg/ml), and stored at -20°C. The stock solution was further diluted in water 1:100 to give a working solution of 0.01 mg/ml. 500 µl aliquots stored at -20°C.

Interleukins 1β (IL-1β), 4 (IL-4), 13 (IL-13): 5 µg IL-1β, IL-4 or IL-13 (R&D Systems, UK) dissolved in 5 mls PBS (Dako, UK) containing 0.1% w/v bovine serum albumin (final concentration 1 ng/µl). 500 µl aliquots stored at -20°C.

Lipopolysaccharide (LPS): 10 mg LPS (derived from Escherichia coli serotype 0111:B4) solubilised in 5 ml sterile 0.9% NaCl and stored in 200 µl aliquots at -20°C. Immediately prior to use, diluted further 1:4 in 0.9% NaCl to give a working concentration of 0.5 mg/ml.

Norepinephrine (NE, Bitartrate salt, FW 319.3): 31.93 mg NE dissolved in 10 mls distilled water (final concentration 10^{-2} M). 500 µl aliquots stored at -20°C.

Tumour necrosis factor α (TNFα): 10 µg TNFα (R&D Systems, UK) dissolved in either 1 ml PBS (Dako, UK) containing 0.1% bovine serum albumin (final concentration 10 ng/µl) or in 10 mls PBS (Dako, UK) containing 0.1% bovine serum albumin (final concentration 1 ng/µl). Aliquots stored at -20°C.

2.1.3 Drugs used in clinical studies

Acetylcholine (ACh): 1 ml of 10 mg/ml pharmaceutical grade ACh (Cibavision Ophthalmics, Southampton, UK) dissolved in 500 ml 0.9% NaCl immediately prior to use and diluted further to give final concentrations of 5, 10 and 20 µg/ml.

Bradykinin (BK, FW 1060.2): 52 µg pharmaceutical grade BK (Clinalfa, Läufelfingen, Switzerland) dissolved in 50 ml 0.9% NaCl immediately prior to use and diluted further to give final concentrations of 100, 300 and 1000 pmol/ml.

Hydrocortisone: 100 mg pharmaceutical grade hydrocortisone (Solu-Cortef®; Pharmacia and Upjohn, UK) dissolved in 100 ml 0.9% NaCl immediately prior to use and diluted further to give final concentrations as described in the study protocol.

Metyrapone: 750 mg (3 x 250 mg capsules, Metopirone®; Alliance, UK) administered orally according to study protocol.

Saline (NaCl): 0.9% w/v NaCl (Baxter, UK) containing 154 mM sodium and 154 mM chloride.

Sodium Nitroprusside (SNP): 50 mg pharmaceutical grade sodium nitroprusside (David Bull Laboratories, Faulding, UK) dissolved in 500 ml 0.9% NaCl immediately prior to use and diluted further to give final concentrations of 2, 4 and 8 μg/ml. Stored in light-protected syringes.

2.2 Animals

Male C57B6J mice were obtained from Charles River, Kent, UK. Leptin deficient *ob/ob* mice on a C57B6J genetic background and Wistar rats were purchased from Harlan Orlac, UK. 11βHSD 1 homozygous null (-/-) and 11βHSD 2 homozygous null (-/-) mice were bred in-house at the Biomedical Research Facility, Western General Hospital, Edinburgh, UK. Genetic inactivation of 11βHSD1 and 11βHSD2 have been described previously in MF-1/129 mice (Kotelevtsev *et al.* 1997; Kotelevtsev *et al.* 1999); for the current experiments mice were backcrossed over more than 10 generations onto a C57B6J background (Morton *et al.* 2004a).

Animals were maintained under controlled conditions of light (on 0800 to 2000h) and temperature (21°C) with free access to standard chow (Special Diet Services, Witham, UK) and water. Animal experiments were carried out under Home Office licence and conformed to standards defined in "The Principals of Animal Care" (NIH publication no. 85-23, revised 1985). Mice were killed by either cervical dislocation or decapitation; rats were killed by exposure to CO_2.

2.3 Intact vessel preparation

Mice aged 8-16 weeks were killed by cervical dislocation. The thoraco-abdominal aortae and proximal hindlimb vasculature were immediately removed into ice-cold DMEM-F12 culture medium and cleaned of periadventitial fibroadipose tissue. Liver and kidneys were also removed as intact organs into ice-cold DMEM-F12 culture medium, and sliced into segments weighing approximately 25-50 mg for immediate use as control tissues in enzyme activity assays. For homogenate assays, aortae were snap frozen on dry ice and then stored at -80°C. For smooth muscle cell isolation, aortas were kept whole and used immediately. For *in vitro* intact tissue assays, aortae were divided into 2-3 mm rings and the proximal hindlimb vessels (external iliac and femoral arteries) were divided into proximal and distal segments, and used immediately.

2.4 Tissue homogenisation

Whole, frozen aortae were crushed in chilled tin foil under liquid nitrogen, using a mortar and pestle, and then mechanically homogenised in 0.4 mls ice-cold Krebs-Ringer buffer using an Ystral mechanical homogenizer (Scientific Instruments Centre, UK). Homogenates were kept on ice for 10-15 min to allow any remaining solid tissue to sink, and the supernatant was removed. A 40 µl aliquot from each homogenate was removed for immediate protein assay. Remaining samples were stored at -80°C prior to assay.

2.5 Protein assay

The protein concentration of aortic homogenates was determined using a colorimetric Bio-Rad protein assay kit (Bio-Rad, Hertfordshire, UK). A range of standards (0.02–0.3 mg/ml) was prepared in duplicate in distilled water from the provided protein standard (bovine serum albumin). Protein assay dye reagent was diluted 1:4 in distilled water and filtered through Whatman No. 1 filter paper prior to use. Diluted protein assay dye reagent (1.96 ml) was added to 40 µl of protein standard or appropriately diluted tissue homogenate in a borosillicate tube, vortexed

to mix and left at room temperature for 15 min–1 hour to allow colour development. Absorbance of samples at λ595nm was measured using a Shimadzu UV/ visible recording spectrophotometer and the concentration of protein in each sample was estimated from the standard curve.

2.6 Synthesis of [^{3}H]-11-dehydrocorticosterone

120 µl 1,2,6,7-[^{3}H]-corticosterone was dried down under nitrogen gas at 60°C and reconstituted in 50 µl 100% ethanol. Reconstituted 1,2,6,7-[^{3}H].corticosterone was incubated in a glass vial at 37°C with 300 µl placental homogenate and 200 µl NAD cofactor (25 mM) in a final volume of 5 mls C buffer. After 4 hours, steroids were extracted by serial additions of 8 mls ethyl acetate to a total of 10 volumes. After each addition, the sample was vortexed and then centrifuged at 1700 *g* for 10 min at 4°C. The upper organic layer was transferred to a fresh tube, dried down under nitrogen, reconstituted in 100 µl ethanol and stored at –20 until use. A 1 µl aliquot of the 1,2,6,7-[^{3}H].corticosterone product was analysed by HPLC for assessment of purity and concentration.

2.7 Smooth muscle cell culture

Murine aortic smooth muscle cells (MA-SMCs) were isolated and cultured by modification of a method by Ray *et al.* (2001). Following aortic preparation as described in Section 2.3, single aortas were incubated in modified Hanks' balanced salt solution (HBSS, Invitrogen, containing 1% Penicillin/Streptomycin and 0.1% Fungizone; Gibco) containing 175 U/ml Collagenase Type 2 (Worthington) for 3 to 5 minutes. The adventitia was separated from the aortae by dissection, the vessel was opened longitudinally, and the endothelial cell layer removed by friction with blunt forceps. Aortae were cut into 1–2 mm pieces and incubated overnight at 37°C in oxygenated (95% O_2:5% CO_2) DMEM (Gibco) containing 10% Foetal Calf Serum (FCS; Gibco). Aortae were then transferred to medium containing 420 U/ml Collagenase type 2 and incubated for a further 3-4 hours. The cells were suspended in culture medium (DMEM containing 20% FCS, 1% Penicillin/ Streptomycin and

1% L-Glutamine 200mM; Gibco), centrifuged twice at 300 *g* for 5 mins and then resuspended in culture medium in a humidified oxygenated (95% O_2:5% CO_2) atmosphere.

The cells were confirmed as smooth muscle cells by staining with α-actin (Sigma), using human umbilical vein endothelial cells (HUVECs) as a negative control. Cells were counted on a haemocytometer, using a 1 in 10 dilution with Trypan Blue (Sigma).

Cells were maintained in DMEM (containing 10% FCS, 1% Streptomycin, 1% Penicillin and 1% L-Glutamine) in 75 cm^2 flasks in a humidified oxygenated (95% O_2:5% CO_2) atmosphere. Cells were passaged once they reached confluence (approximately once weekly). Cells at their 2^{nd} passage were used for activity assays unless otherwise stated.

2.8 Measurement of 11βHSD activity *in vitro*

Although 11βHSD1 is a predominant reductase *in vivo* (catalysing the conversion of inactive 11-dehydrocorticosterone to active corticosterone (Jamieson *et al.* 2000)), in homogenised cell preparations it is capable of bi-directional activity, with the dehydrogenase direction predominating (Lakshmi & Monder 1988). Thus, conventional *in vitro* assays have measured 11βHSD1 activity in the dehydrogenase direction using homogenised tissue preparations. This technique is limited as it is not possible to differentiate between activities attributable to the individual isozymes of 11βHSD. In the cell culture and intact tissue assays described below, therefore, 11βHSD activity is measured in both the reductase and dehydrogenase directions.

2.8.1 In cell culture

Murine aortic smooth muscle cells (MA-SMCs) were seeded onto 6 well plates at a density of 1.75 x 10^5 cells per well, in 2 ml of assay medium. The following day, the medium was changed to basal medium containing minimal (0.5%) FBS. After 48 hours, 10 pmol [^{3}H]-11-dehydrocorticosterone was added to the appropriate wells

and cells were further incubated for 24 hours. After incubation, the overlying culture medium was removed to a glass tube and stored at -20°C prior to Sep Pak steroid extraction.

Enzyme activity was expressed as conversion per 10^5 cells after subtraction of apparent conversion in negative control wells.

2.8.2 In homogenised aortae

11β-Dehydrogenase activity was measured in homogenates of aortae from *ob/ob* and littermate control mice by a method adapted from Livingstone *et al.* (2000b). Homogenised aortic tissue was prepared in duplicate in a total volume of 250 µl Krebs-Ringer buffer containing 0.2% glucose, NADP (2 mM) and [^{3}H]-corticosterone (100 nM). Final protein concentration was 20 µg /ml. Samples containing no cofactor served as negative controls, and tissue blanks were prepared in duplicate in KRB buffer with NADP and [^{3}H]-corticosterone in the absence of tissue homogenate. Samples were incubated for 24 hours at 37°C. Following incubation, the reaction was stopped by the addition of 10 volumes of ethyl acetate to each tube, and tubes were vortexed. The upper organic phase (containing steroids) was removed to a fresh tube and evaporated under oxygen free nitrogen at 60°C.

Enzyme activity was expressed as percentage conversion per 5 µg protein after subtraction of apparent conversion in negative control samples.

2.8.3 In intact vascular preparations *in vitro*

11β-reductase and –dehydrogenase activities were measured in aortic rings and hindlimb vasculature from C57B6J, 11βHSD1 homozygous null (-/-) and 11βHSD2 homozygous null (-/-) mice by adaptation of a method by Souness *et al.* (2002). Aortic rings were incubated for 24 hours at 37°C in 1 ml of DMEM-F12 medium containing [^{3}H]-steroid supplemented with streptomycin (100 µg/ml), penicillin (100 units/ml) and amphotericin (0.25 µg/ml). 11β-Reductase activity was determined by

adding 10 pmol [^{3}H]-11-dehydrocorticosterone. Murine liver slices incubated under the same conditions served as a positive control. 11β-Dehydrogenase activity was determined by adding 10 pmol [^{3}H]-corticosterone. Murine kidney slices served as a positive control in this case. As negative controls, [^{3}H]-11-dehydrocorticosterone or [^{3}H]-corticosterone was incubated in wells without tissue, and wells containing medium alone served as blanks. Experiments were performed in duplicate (and triplicate when possible). Following incubation, the overlying culture medium was removed to a glass tube and stored at -20°C prior to Sep Pak extraction of steroids. Tissue samples were blotted and weighed. It has previously been shown that aortic ring tissue contains only 2-3% of the added radioactivity so that only the supernatant, and not the vessels themselves, was included in the extraction (Souness *et al.* 2002).

Enzyme activity was expressed as percentage conversion per wet weight of aortic tissue per 24 hours after subtraction of apparent conversion in negative control samples.

2.9 Hindlimb perfusion model

2.9.1 Hindlimb set-up

The method used was an adaptation from that of Brandes *et al.* (2000). After sacrifice by cervical dislocation, a laparotomy incision was performed and the aorta and inferior vena cava (IVC) at the thoraco-abdominal transition was prepared by blunt dissection. 6/0 Prolene sutures (Surgical Supplies Ltd, Cumbernauld, UK) were threaded under the distal aorta and inferior vena cava (IVC), just proximal to the aortic bifurcation, and under the proximal abdominal aorta and IVC, distal to the renal and testicular arteries. A 24-gauge cannula (Neoflon, Ohmeda, Sweden) was inserted into the aorta distal to the renal and testicular arteries and advanced to the aortic bifurcation. An 18-gauge cannula (Venflon, BD, UK) was inserted into the abdominal IVC, distal to the renal veins and advanced distally to a similar level of the arterial cannula. Both cannulae were tied in with the distal suture to prevent leakage and retrograde perfusion. The proximal suture was used to tie off both the aorta and IVC distal to the renal and testicular vessels, to prevent retrograde

perfusion of abdominal viscera, and then tied around both cannulae. A schematic diagram of the hindquarter perfusion technique is shown in Figure 2.1

2.9.2 Perfusion technique

Modified Krebs-Henseleit buffer supplemented with 2% BSA was maintained in a water bath at 40°C, whilst constantly bubbled with carbogen (95% O_2:5% CO_2). A peristaltic pump was used to constantly perfuse the hindquarters with warmed oxygenated buffer at a rate of between 0.8 and 1.2 ml/min. Hindquarters were perfused with oxygenated buffer within 30 minutes of sacrifice, and perfusion studies lasted up to 60 minutes.

2.9.3 11βHSD activity

Following a ten minute equilibration period, [^{3}H]-11-dehydrocorticosterone (for determination of reductase activity) or [^{3}H]-corticosterone (for determination of dehydrogenase activity) was added to the perfusion buffer at a concentration of 5 nM. Aliquots of effluent were collected at intervals throughout the perfusion and stored at -20°C until analysis.

Steroids were purified from the effluent samples by Sep Pak extraction and analysed by HPLC (as described in Section 2.10). Buffer containing [^{3}H]-11-dehydrocorticosterone or [^{3}H]-corticosterone served as a positive control, and buffer containing no radioactivity served as a negative control.

2.9.4 Effect of systemic inflammation on 11β-reductase activity

To observe the effects of inflammation on 11βHSD activity in the perfused hindlimb, lipopolysachharide (LPS, 10 mg/kg in a volume of 20 ml/kg) or 0.9% saline vehicle was administered to C57B6J mice by intraperitoneal injection at 0800 h. 6 hours later, mice were sacrificed by cervical dislocation and underwent a hindlimb perfusion protocol as described above. On each occasion, an LPS-treated and vehicle-treated mouse were studied on the same day.

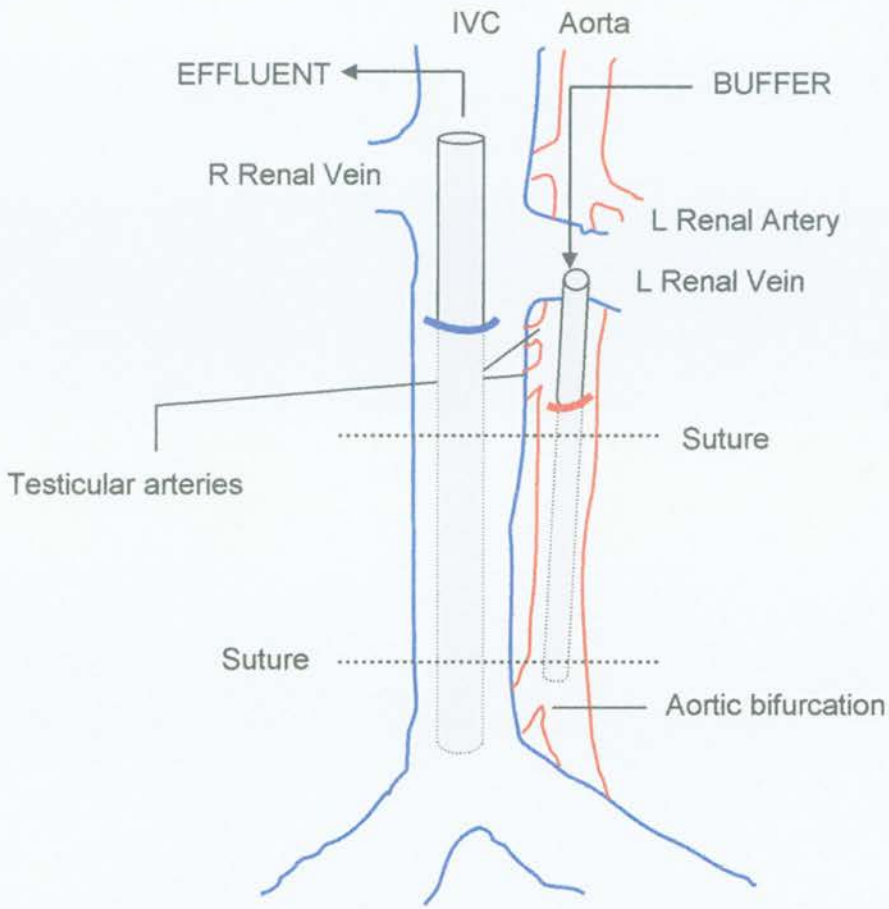

Figure 2.1 Schematic diagram of murine hindquarter perfusion technique

Following laparotomy, 24-gauge and 18-gauge cannulae were inserted into the aorta and inferior vena cava (IVC), respectively. The aortic cannula was inserted distal to the renal and testicular arteries and advanced to the aortic bifurcation. The IVC, cannula was inserted distal to the renal veins and advanced distally to a similar level of the arterial cannula. To prevent retrograde flow, sutures were tied around both vessels (i) below the level of the testicular arteries, and (ii) proximal to the aortic bifurcation as shown. Buffer was perfused into the aortic cannula, and effluent collected from the inferior vena cava cannula, as described in detail in the Methods Section 2.9.2.

2.9.5 Systemic effects of lipopolysaccharide

In a further set of studies to characterise the systemic response to LPS, C57B6J mice underwent LPS or vehicle administration as described in Section 2.9.4. Mice were weighed prior to injection and prior to sacrifice. 6 hours following drug administration, mice were killed by decapitation and aortae were removed into ice-cold DMEM in preparation for *in vitro* activity assays. The spleens were weighed, snap frozen and stored at -80°C.

2.10 Steroid analysis

2.10.1 Sep Pak extraction and purification

In order to extract a clean preparation of steroids from culture medium or buffer, samples were run through C_{18} Sep Pak columns (Waters Millipore, Watford, UK) under gravity. The columns were prepared for use with 5 mls HPLC grade methanol, to separate the C_{18} chains, and then washed with 5 mls HPLC grade H_2O. The sample was then run onto the column, washed once with 5 mls HPLC grade H_2O and eluted into glass tubes using 2 mls HPLC grade methanol. Steroid-containing methanol samples were then dried down at 60°C under nitrogen gas.

2.10.2 High Performance Liquid Chromatography

The high-performance liquid chromatography (HPLC) system comprised an auto-sampler and mobile phase pump (Waters, UK), a symmetry shield RP_{18} 5 μm column (Waters, UK) and a radioactivity monitor linked to a scintillation fluid pump (Berthold, UK). The system was controlled by the Winflow computer programme (JMBS Developments, France). Steroid samples extracted by either ethyl acetate (in the case of tissue homogenates or cell culture) or Sep Pak extraction (in the case of intact vascular tissue experiments) were re-suspended in 1 ml mobile phase (60% water, 15% acetonitrile, 25% methanol) and 180 μl of each sample was injected into the HPLC system. The flow rate of the mobile phase was 1 ml/ min and the flow rate of the scintillant (Quicksafe Flow 2; Zinsser, UK) was 2 ml/ min to achieve optimal

mixing and counting efficiency. The column temperature was 35°C to improve peak shape and maintain stability of retention times. Radioactive standards were injected at the start of each batch of samples to confirm peak identity. The approximate retention times for [^{3}H]-corticosterone and [^{3}H]-11-dehydrocorticosterone were 12-14 min and 16-19 min respectively (variations in retention times in some assays were due to intentional changes in mobile phase composition and column condition), with greater than 1 minute between the two peaks. Peaks were less than 1min 30s wide and peak height was at least 50-fold higher than background. An additional peak (retention time 9-10 min, always less than 6% of total radioactivity) was present in a proportion of samples, particularly those from assays involving a long incubation duration or in samples from assays using 11βHSD1 -/- mice. By exclusion of other possible metabolites, it was determined that this peak may be the 20β-dihydrocorticosterone metabolite of corticosterone. However, standards of this steroid metabolite are unavailable to allow confirmation of this. A representative chromatogram obtained from a typical assay is shown in Figure 2.2. Following chromatography, the area under each peak was integrated using the Winflow software and used to quantify the percentage conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone (11β-dehydrogenase activity) or [^{3}H]-11-dehydrocorticosterone to [^{3}H]-11-corticosterone (11β-reductase activity). The percentage conversion in each tissue sample was corrected for the "apparent conversion" occurring in tissue blanks included in each experiment, which was always <2%.

2.11 TNFα bioactivity assay

To confirm bioactivity of the murine TNFα, isolated human neutrophils ($5x10^6$ cells per well) were incubated in a volume of 150 μl containing 12.5 ng/ml TNFα for 2-6 hours (Murray *et al.* 1997). 100 μl of the cells were plated onto glass slides and stained with Diff Quick (Baxter Healthcare, UK). Cells were examined under oil immersion light microscopy, and the proportion of apoptotic neutrophils (defined as those containing darkly stained pyknotic nuclei (Savill *et al.* 1989)) was estimated. The results are shown in figure 2.3.

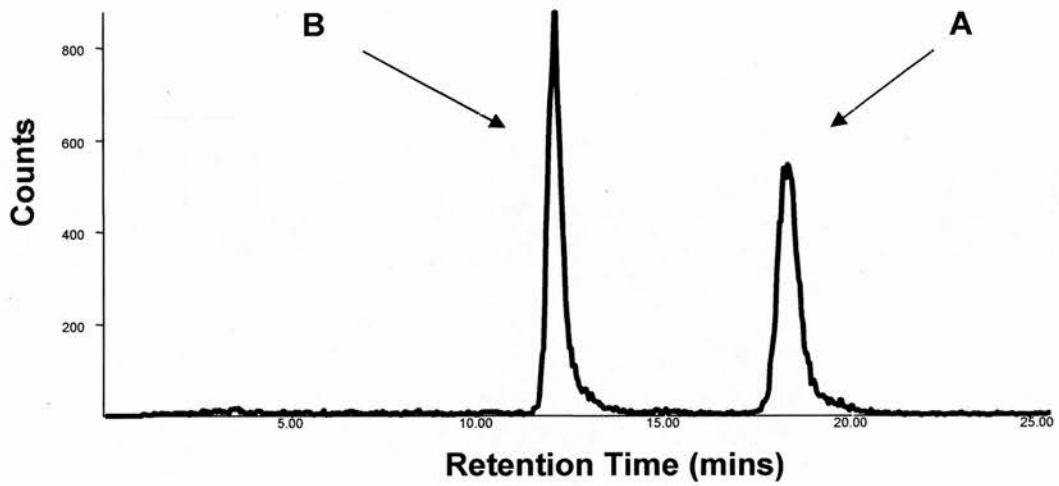

Figure 2.2 Representative HPLC chromatogram

A representative chromatogram from high performance liquid chromatography of a sample from an 11β-reductase assay. Retention times for [^{3}H]-11-dehydrocorticosterone (A) and [^{3}H]-corticosterone (B) were compared with those of known standards.

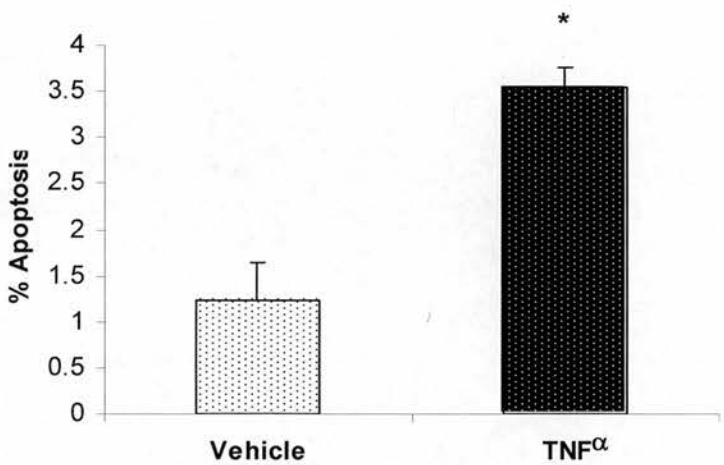

Figure 2.3 Neutrophil apoptosis assay to demonstrate TNFα bioactivity

A human neutrophil apoptosis assay was used to determine the bioactivity of murine TNFα. Human neutrophils ($5x10^6$ cells per well) were incubated with TNFα (12.5 ng/ml) for 2-6 hours, after which, apoptotic cells were determined by morphological analysis. Results are mean ± standard error, n=3, * $p<0.05$.

2.12 Measurement of endothelial cell function *in vivo* in humans

2.12.1 Subjects

Please refer to Chapter 5, Section 5.2.1

2.12.2 Study design

Please refer to Chapter 5, Section 5.2.2

2.12.3 Drugs

Please refer to Chapter 5, Section 5.2.3

2.12.4 Haemodynamic measurements

Please refer to Chapter 5, Section 5.2.4

2.12.5 Venous sampling

Please refer to Chapter 5, Section 5.2.5

2.12.6 Sample analysis

Please refer to Chapter 5, Section 5.2.6

2.12.7 Data analysis and statistics

Please refer to Chapter 5, Section 5.2.7

2.12 Statistics

Values are expressed as mean ± standard error. Numbers (n) refer to either individual animals or human subjects unless otherwise stated. Data were analysed by Student's t-tests, Analysis of Variance (ANOVA) or ANOVA with repeated measures followed by post-hoc tests as appropriate. Statistical significance was taken at the 5% level.

Chapter Three

Glucocorticoid metabolism in vascular tissue *in vitro*

3.1 Introduction

Modulation of 11βHSD activity may provide a mechanism for local feedback regulation of inflammation in vascular smooth muscle cells. Pro-inflammatory cytokines (e.g tumour necrosis factor-α (TNFα)) up-regulate 11βHSD1 activity and expression and down-regulate 11βHSD2 expression in cultured human aortic smooth muscle cells (Cai *et al.* 2001). These coordinated changes favour an increase in the availability of active glucocorticoid within vascular smooth muscle and would be expected to inhibit inflammation. This is significant, given the importance of inflammation in the vascular response to injury (Wainwright *et al.* 2001). However, whilst cytokine-mediated changes in 11βHSD activity have been demonstrated in cultured vascular cells (Cai *et al.* 2001), adipocytes (Tomlinson *et al.* 2001), macrophages (Thieringer *et al.* 2001), fibroblasts (Sun & Myatt 2003), ovarian epithelial cells (Yong *et al.* 2002; Rae *et al.* 2004), osteoblasts (Cooper *et al.* 2001), and glomerular mesangial cells (Escher *et al.* 1997), the influence of cytokines on intact blood vessels has not been established. Furthermore, it has not been demonstrated whether acute or chronic systemic inflammation (for example in endotoxaemia or obesity respectively) leads to an up-regulation of 11βHSD1 activity in vascular tissue.

In order to determine whether cytokine exposure selectively up-regulates 11βHSD1 in the vascular wall it is necessary to measure activity of both 11βHSD1 and 11βHSD2 isozymes. This is important for two reasons: first, both isozymes are expressed in the vessel wall (although 11βHSD1 may be localised to the smooth muscle (Brem *et al.* 1998) (Christy *et al.* 2003) and 11βHSD2 to the endothelium (Brem *et al.* 1998; Christy *et al.* 2003)) and, second, 11βHSD1 may act in a bi-directional manner (as both a reductase and a dehydrogenase (Brem *et al.* 1995; Souness *et al.* 2002)). In the absence of suitable selective inhibitors for 11βHSD isozymes, mice with genetic inactivation of either 11βHSD1 (Kotelevtsev *et al.* 1997; Morton *et al.* 2004a) or 11βHSD2 (Kotelevtsev *et al.* 1999) provide an invaluable tool for assessing the activities of the individual isozymes. However, whilst these animals have been used to show that both isozymes are active in mouse

aortic homogenates (Hadoke *et al.* 2001; Christy *et al.* 2003) the directionality of 11βHSD1 and 11βHSD2 has not been addressed in intact arteries. The choice of methodology is significant as 11βHSD activity is traditionally measured in the dehydrogenase direction in homogenates (Livingstone *et al.* 2000a; Hadoke *et al.* 2001). However, due to the lack of co-factor specificity between isozymes of 11βHSD in mice (Walker *et al.* 1992b), measurement of both reductase and dehydrogenase activities requires the use of intact tissues (Souness *et al.* 2002).

The work described in this chapter explores the hypothesis that inflammatory mediators selectively up-regulate 11βHSD1 reductase activity in intact blood vessels *in vitro*. The specific aims were (1) to determine the reaction directionality of the 11βHSD isozymes in intact murine arteries *in vitro*, and (2) to investigate the influence of inflammation on 11βHSD activity within cultured mouse aortic smooth muscle cells and in intact murine arteries. These studies required the application of existing techniques and the development of novel methodology to determine 11β-reductase and –dehydrogenase activities in cultured murine aortic smooth muscle cells and in intact murine arteries *in vitro*.

3.2 Methods

3.2.1 Mice

Unless otherwise specified, all arteries were obtained from male C57B6J mice aged 8-16 weeks. Vessels and control tissues were prepared as described in Methods Section 2.3.

3.2.2 Effects of cytokines on 11βHSD activity in cultured murine aortic smooth muscle cells

In order to assess the effects of IL-1β on 11β-reductase activity, cultured murine aortic smooth muscle cells (MA-SMCs, at passage 2; see Section 2.7) were incubated for 48 hours (37°C in 95% O_2:5% CO_2) in 2 ml of basal medium with IL-1β (20

ng/ml) or vehicle. A 48 hour incubation period was chosen as previous studies demonstrated a clear up-regulation of 11β-reductase activity in cultured vascular smooth muscle cells over this period (Cai *et al.* 2001). 11β-Reductase activity was then determined by incubation for a further 24 hours with [^{3}H]-11-dehydrocorticosterone as described in Section 2.8.1. Conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone was determined by HPLC (as described in Section 2.10).

Replication of these studies required the use of cells at later passage stages (4^{th} – 6^{th} passage), and as preliminary experiments suggested that there was considerable variability in basal 11β-reductase in these cells, a reduced cell density (0.5x 10^5 cells per well) was used in later experiments.

To investigate whether the effects of IL-1β on 11β-reductase activity were influenced by corticosterone, 11β-reductase activity was determined in MA-SMCs following prior incubation (48 hours) with IL-1β, corticosterone (100 μM) or both.

To confirm 11βHSD1 as the sole reductase in MA-SMCs, attempts were made to culture MA-SMCs from 11βHSD1-/- mice using the method described in Section 2.7.

3.2.3 11βHSD activity in intact vascular tissue *in vitro*

3.2.3.1 Establishing directionality and enzyme activities in intact tissue

In order to develop a murine *in vitro* model with which to determine the effects of cytokines on vascular 11βHSD activity, extensive development of existing methodology in rat tissue (Souness *et al.* 2002) was undertaken. It was necessary to vary culture conditions, tissue quantities and/or duration of incubations in order to optimise conditions for more detailed investigations.

3.2.3.2 11β-Reductase activity in intact arteries

11β-Reductase activity was determined (as described in Section 2.8.3) in single intact aortic rings and iliofemoral arteries from male C57B6J mice (n=6) using murine liver and kidney as positive control tissues. HPLC analysis (as described in Section 2.10) determined the percentage conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone.

In order to confirm that the standard conditions (as described in Section 2.8.3) were appropriate to measure 11β-reductase enzyme velocity within the linear range, enzyme kinetics were established by repeating the assay using increasing numbers (1-9) of aortic rings per well.

3.2.3.3 11β-Dehydrogenase activity in intact arteries

11β-Dehydrogenase activity was determined in aortic rings and iliofemoral vessels from C57B6J (n=6) mice using murine liver and kidney as a positive control tissues (as described in Section 2.8.3). HPLC analysis (as described in Section 2.10) determined the percentage conversion of [^{3}H]-corticosterone to [^{3}H]- 11-dehydrocorticosterone.

In order to determine the time course of dehydrogenase activity, aortic rings were incubated for 32 hours in medium containing 100 nM [^{3}H]-corticosterone, and 100 μl aliquots of medium were removed at serial time points (n=4).

3.2.4 Effects of selective genetic inactivation of 11βHSD isozymes on 11β-reductase and -dehydrogenase activities

To establish whether 11βHSD1 is the sole reductase in vascular tissue, 11β-reductase activity was determined in single intact aortic rings and iliofemoral arteries from male C57B6J and homozygous null 11βHSD1 (-/-) mice, using liver and kidney slices as control tissues (as described in Section 2.8.3; n=6).

To determine whether 11βHSD1 exhibits dehydrogenase activity in intact vascular tissue, 11β-dehydrogenase activity was determined in aortic rings, and kidney slices from male C57B6J and homozygous null 11βHSD2 (-/-) mice (as described in Section 2.8.3; n=4).

3.2.5 Influence of cytokines on 11βHSD activity in intact arteries *in vitro*

To examine the effects of pro-inflammatory cytokines on 11βHSD activity, 11β-reductase and –dehydrogenase activities were determined (as described in Section 2.8.3) in aortic rings from C57B6J mice following incubation for 16 hours (37°C, 95%:5% O_2:CO_2) with IL-1β (10 ng/ml), TNFα (100 ng/ml) or vehicle (n=4-10). In a previous study of cultured aortic SMCs, TNFα exerted maximal effect on 11β-reductase activity after 16 hours (Cai *et al.* 2001), hence this incubation period was chosen. To establish whether there was a dose-response to these inflammatory cytokines, further studies were performed using a range of concentrations of IL-1β (1-100 ng/ml) and TNFα (10-1000 ng/ml).

Other cytokines which have been reported to influence 11βHSD1 activity include the T-lymphocyte derived interleukins, IL-4 and IL-13 (Thieringer *et al.* 2001). To investigate whether these cytokines influence 11βHSD1 activity in intact vessels, 11β-reductase activity was determined in aortic rings following incubation (16 hours) with either IL-4 (50 ng/ml) or IL-13 (50 ng/ml).

In order to examine whether endogenous inflammation was contributing to regulation of basal 11βHSD activity within the intact aortic ring, 11β-reductase assays were performed in aortic rings following incubation (16 hours) with etanercept (a fusion protein that antagonises human and murine TNFα (Xing, L, Personal communication), and which ameliorates the cardiovascular effects of murine TNFα (Vallejo *et al.* 2005); 0.1-10 μg/ml; n=4).

3.2.6 Influence of systemic lipopolysaccharide *in vivo*

In order to investigate the influence of *in vivo* inflammation on 11βHSD activity, 11β-reductase and –dehydrogenase activities were determined (as described in Section 2.8.3) in aortic rings from C57B6J mice which had received intraperitoneal lipopolysaccharide (LPS, 10 mg/kg) or saline 6 hours previously (as described in Section 2.9.5; n=3-6).

3.2.7 11βHSD activity in aortic homogenates from obese *ob/ob* mice

Obesity is characterised by systemic inflammation (Rajala & Scherer 2003) and up-regulation of adipose 11βHSD type 1 activity in both humans (Sandeep *et al.* 2005) and rodents (Livingstone *et al.* 2000a). The possibility that obesity, as a chronic inflammatory state, might alter 11βHSD activity within vascular tissue was explored in aorta from obese *ob/ob* mice. Total 11βHSD activity was determined by measurement of dehydrogenase activity in homogenised preparations of aorta (as described in Section 2.8.2) from obese *ob/ob* and lean littermate control mice (n=8-11). HPLC analysis (as described in Section 2.10) determined the percentage conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone by aortic homogenates with known protein concentrations.

3.2.8 Statistics

Data are expressed as mean ± standard error and were analysed by Student's t-test or ANOVA and post-hoc tests where appropriate.

3.3 Results

3.3.1 Effects of cytokines on 11βHSD activity in cultured murine aortic smooth muscle cells

Stimulation with IL-1β significantly increased 11β-reductase activity (87±2% conversion compared with 62±3% in controls, $p<0.05$, n=6; Figure 3.1) in cultured MA-SMCs (n=6). Replication of these studies, however, showed the basal activity to be highly variable (data not shown). Furthermore, the up-regulation of 11β-reductase activity by IL-1β was difficult to reproduce (Figure 3.2). On further investigation, two variables had a marked influence on reductase activity: passage stage and the presence of corticosterone. Basal 11β-reductase activity in MA-SMCs was lower in cells at higher passage stage (n=6, Figure 3.2). Also, in experiments in which 11β-reductase activity in MA-SMCs was either unchanged or only modestly up-regulated upon stimulation with IL-1β, co-incubation with exogenous corticosterone (100 μM) resulted in a more marked (80%) increase in 11β-reductase activity in response to the addition of IL-1β (Figure 3.3).

Despite several attempts to culture murine aortic smooth muscle cells from 11βHSD type 1 homozygous (-/-) null mice using the same conditions as for C57B6J MA-SMCs, there was no evidence of cell growth.

3.3.2 11βHSD activity in intact arteries *in vitro*

3.3.2.1 11β-Reductase activity

11β-Reductase activity was present in intact aortic rings, hindlimb arteries and liver and kidney slices from wild type C57B6J mice, as indicated by conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone (Figure 3.4). In wild type tissues, 11β-Reductase activity was higher in aorta as compared with hindlimb arteries (n=6, $p<0.005$), and both vascular tissues had higher reductase activity when compared with the control tissues (n=6, $p<0.005$).

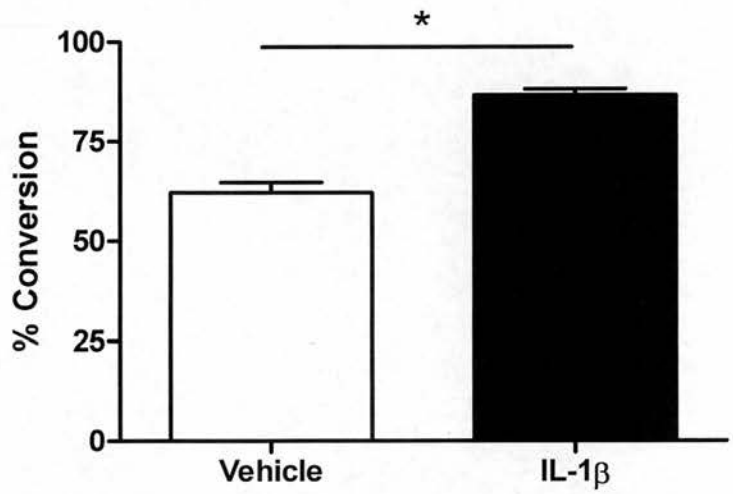

Figure 3.1 Effect of IL-1β on 11β-reductase activity in cultured MA-SMCs

11βHSD activity is expressed as the percentage conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone by 1.75 x 10^5 cells (passage 2) from C57B6J mice. 11βHSD activity was measured after 24 hour incubation. 48 Hour stimulation with IL-1β significantly increased 11β-reductase activity (87±2% conversion compared with 62±3% in controls, * $p<0.05$ when compared by unpaired Student's t-test). Results are mean ± standard error, n=6 (where n refers to the number of wells).

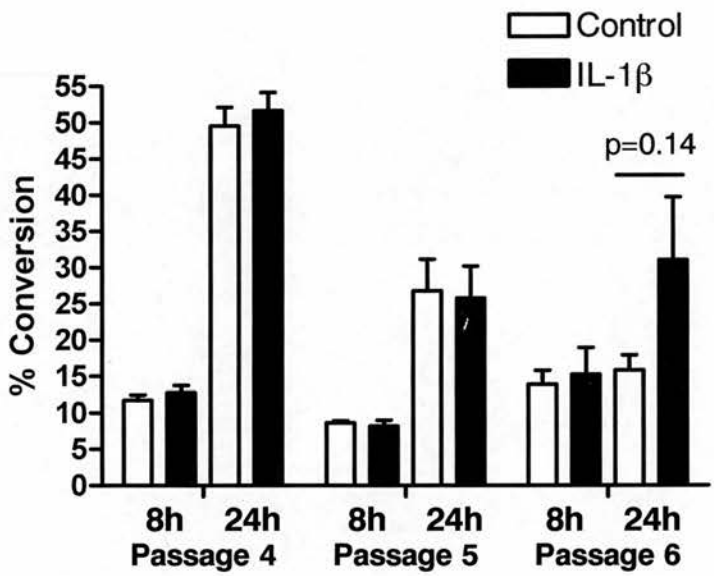

Figure 3.2 Effect of passage on 11β-reductase activity in cultured MA-SMCs

11βHSD activity is expressed as the percentage conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone by 0.5 x 10^5 cells from C57B6J mice. 11βHSD activity was measured at 8 and 24 hours, with (solid bars) or without (open bars) prior incubation (48 hours) with IL-1β (20 ng/ml). Results are mean ± standard error, n=6 (where n refers to the number of wells).

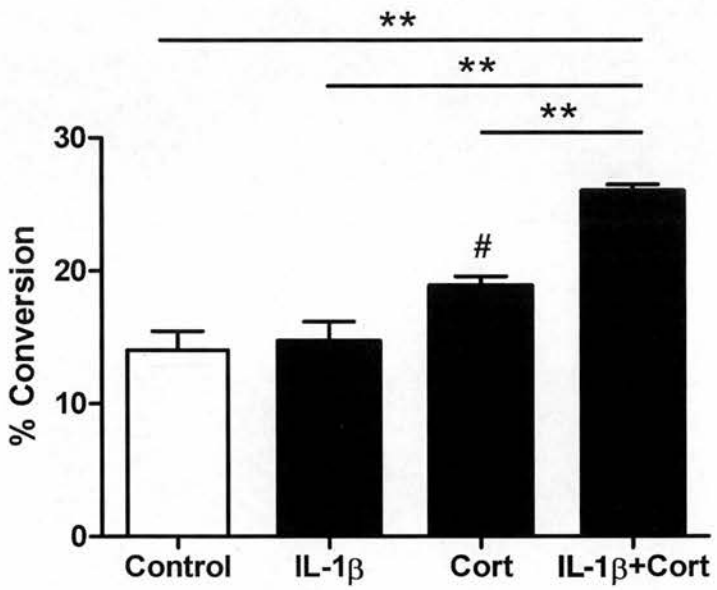

Figure 3.3 Influence of IL-1β and corticosterone on 11β-reductase activity in cultured MA-SMCs

11βHSD activity is expressed as the percentage conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone by 0. 5 x 10^5 cells from C57B6J mice. 11βHSD activity was measured at 24 hours, following prior incubation (48 hours) with IL-1β (20 ng/ml), corticosterone (100 μM) or both. 11β-Reductase activity was significantly increased, compared with control, following corticosterone incubation ($^{\#}p<0.05$). Combined incubation with IL-1β and corticosterone resulted in significantly higher 11β-reductase activity compared with control, IL-1β alone and corticosterone alone, when analysed by ANOVA and post-hoc analysis (**$p<0.001$). Graph shows combined data using cells at passage 1 (n=3 wells) and 2 (n=6 wells). Results are mean ± standard error.

Reductase activity within liver and kidney slices were not significantly different under these assay conditions (n=6, p=0.18). A linear relationship between wet tissue weight and reductase activity in aortic tissue was demonstrated, confirming that, by using single aortic rings in subsequent assays, any increase in activity should be readily detectable (Figure 3.5).

3.3.2.2 11β-Dehydrogenase activity

11β-Dehydrogenase activity was present in intact aortic rings, hindlimb arteries and liver and kidney slices from C57B6J mice, as indicated by conversion of [^{3}H]-corticosterone to [^{3}H]- 11-dehydrocorticosterone (Figure 3.6). 11β-Dehydrogenase activity was similar in aortic rings and hindlimb arteries (n=6, p=0.48). Although the reductase (Figure 3.4) and dehydrogenase (Figure 3.6) assays were not performed contemporaneously, it is worthwhile noting that the absolute levels of reductase activity in vascular tissue were higher than the dehydrogenase activity, suggesting that the predominant direction of 11βHSD activity within vascular tissue is in the reductase direction.

The generation of product over time by dehydrogenase activity in aortic rings and kidney and liver slices is shown in Figure 3.7. In all three tissues, a plateau of activity was reached by 32 hours. In aortic rings and kidney slices, there was an initial linear phase of product generation which persisted beyond 24 hours (Figure 3.7A and 3.7B respectively). However, there was a biphasic activity in liver slices (Figure 3.7C) with a rapid initial rate, which fell again before a more gradual rise to a plateau.

As dehydrogenase activity is much lower than reductase activity in aortic tissue, optimal assay conditions were chosen for subsequent investigations (2-5 aortic rings per well, incubated for 24 hours) to ensure that it would be possible to detect both basal enzyme activity, as well as the predicted decrease in activity following inflammatory stimulation.

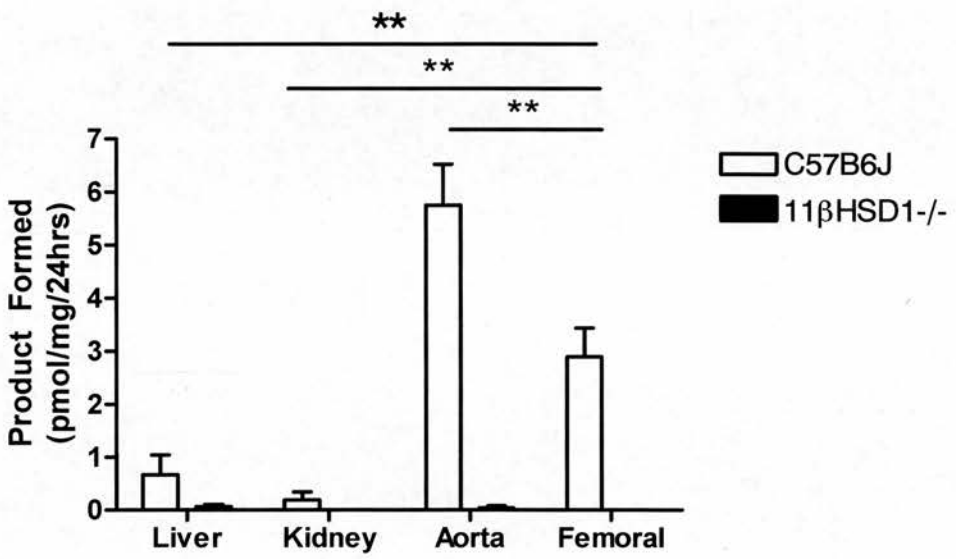

Figure 3.4 Effect of 11βHSD type 1 deletion on 11β-reductase activity in murine tissues

11β-Reductase activity is expressed as the amount of [^{3}H]-corticosterone formed from [^{3}H]-11-dehydrocorticosterone, per milligram (wet weight) over 24 hours, in liver slices, kidney slices, aortic rings and hindlimb artery segments from C57B6J (open bars) and 11βHSD type 1 homozygous null (-/-; solid bars) mice. 11β-Reductase activity in the control tissues from C57B6J mice was similar ($p=0.18$ when compared by unpaired Student's t-test). Reductase activity was higher in the wild type aortic tissue compared with the femoral tissue (**$p<0.005$) and both vascular tissues demonstrated higher activity than control tissues (**$p<0.005$). Reductase activity was effectively abolished in tissues from 11βHSD type 1 -/- mice (activity less than 0.1 pmol/mg/24hrs). Results are mean ± standard error, n=6.

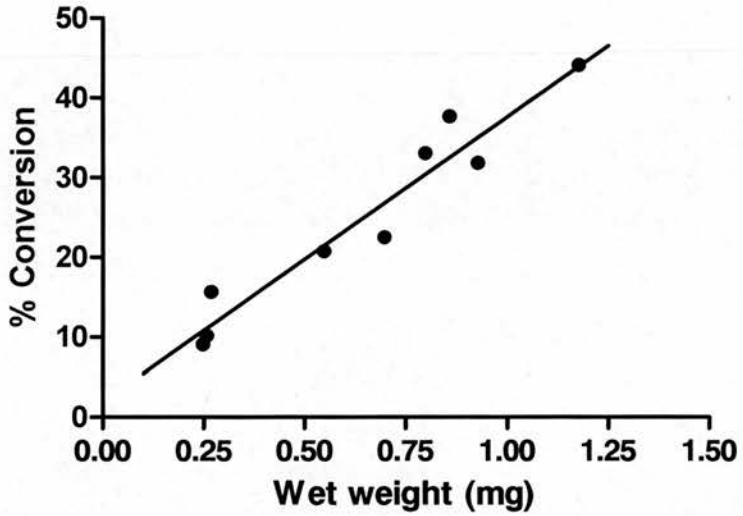

Figure 3.5 11β-Reductase activity in aortic rings: a comparison of activity by wet weight of tissue

11β-Reductase activity in aortic rings from C57B6J mice is expressed as the percentage of [^{3}H]-corticosterone formed from [^{3}H]-11-dehydrocorticosterone over 24 hours. The trendline shown has the equation y = 35.655x + 1.8934, with an R^2 value of 0.9313. Results are for single wells containing 1, 3 or 6 rings each. Representative graph from an experiment repeated twice, using aorta from 6 mice.

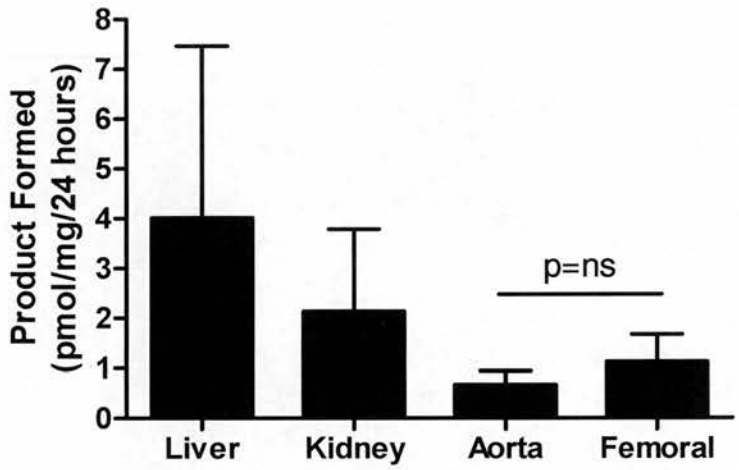

Figure 3.6 11β-Dehydrogenase activity in tissues from C57B6J mice

11β-Dehydrogenase activity is expressed as the amount of [^{3}H]-11-dehydrocorticosterone formed from [^{3}H]-corticosterone, per milligram (wet weight) over 24 hours, in liver slices, kidney slices, aortic rings and hindlimb vascular segments from C57B6J mice. 11β-Dehydrogenase activity did not differ significantly between vascular tissues (p=0.48 when compared by unpaired Student's t-test). Results are mean ± standard error, n=6.

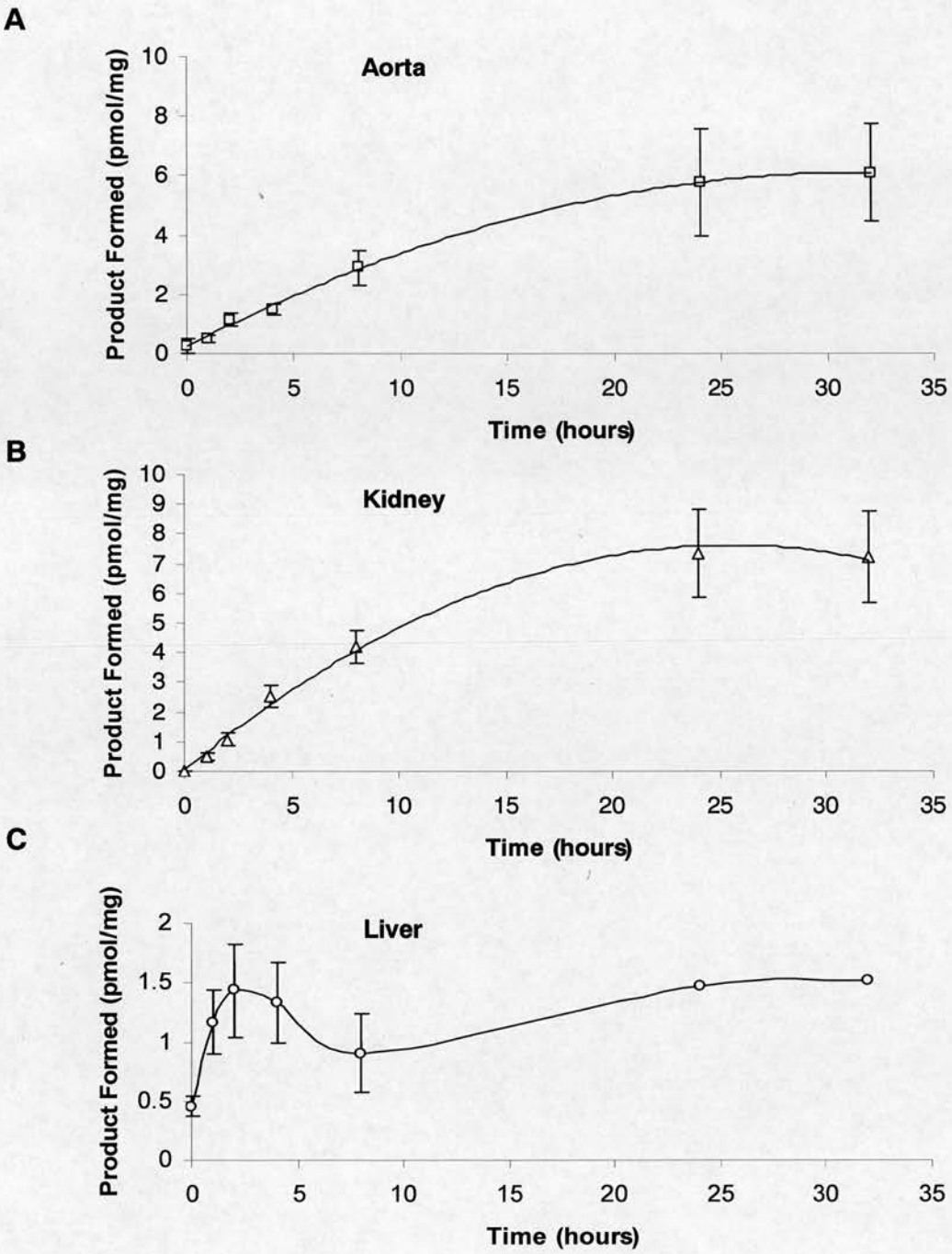

Figure 3.7 11β-Dehydrogenase activity as a function of time

11β-Dehydrogenase activity is expressed as the amount of [^{3}H]-11-dehydrocorticosterone formed from [^{3}H]-corticosterone per milligram tissue, over time, in (**A**) aortic rings, (**B**) kidney slices and (**C**) liver slices from C57B6J mice. Results are mean ± standard error, n=4 (except final two liver time points which are n=2).

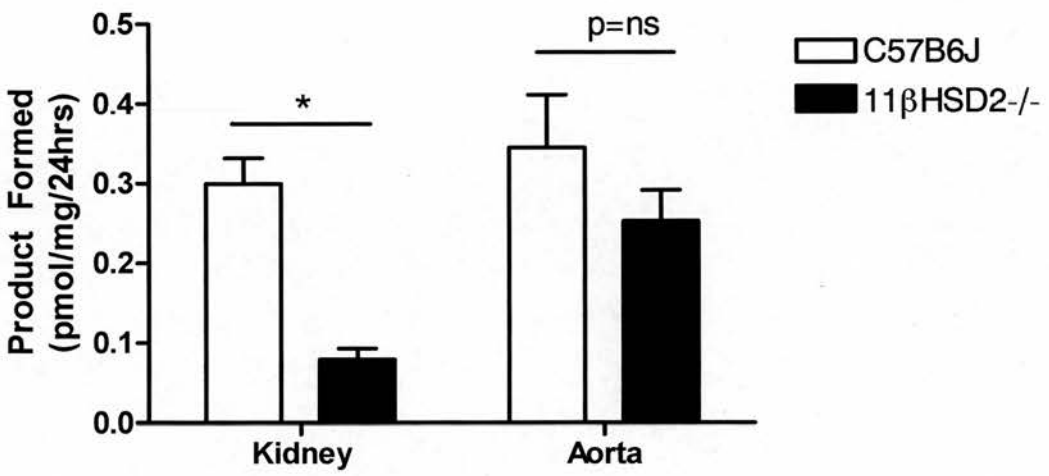

Figure 3.8 11β-Dehydrogenase activity in tissues from 11βHSD type 2 homozygous null (-/-) mice

11β-Dehydrogenase activity is expressed as the amount of [^{3}H]-11-dehydrocorticosterone formed from [^{3}H]-corticosterone, per milligram (wet weight) over 24 hours, in kidney slices and aortic rings from C57B6J and 11βHSD type 2 homozygous null (-/-) mice (n=4). 11β-Dehydrogenase activity was significantly reduced in kidney (*$p<0.005$) but not in aorta ($p=0.28$) from 11βHSD 2 -/- mice compared with controls. Results are mean ± standard error, and were compared by unpaired Student's t-test.

3.3.3 Effects of selective genetic inactivation of 11βHSD isozymes on 11β-reductase and dehydrogenase activities

Genetic inactivation of 11βHSD1 effectively abolished 11β-reductase activity in all tissues studied, with all activity less than 0.1 pmol/mg/24hrs (n=6, Figure 3.4). By contrast, genetic inactivation of 11βHSD2 resulted in a marked reduction, but not abolition, of 11β-dehydrogenase activity in kidney (n=4, $p<0.005$), and did not significantly reduce 11β-dehydrogenase activity in aortic rings ($p=0.28$, n=4, Figure 3.8).

3.3.4 Influence of cytokines on 11βHSD activity in intact arteries *in vitro*

Pre-incubation of aortic rings with the pro-inflammatory cytokines IL-1β and TNFα did not alter 11β-reductase activity (n=4-10, Figure 3.9). Similarly, there was no effect of the Th2 cytokines, IL-4 or IL-13, or of etanercept on reductase activity (n=4, Figure 3.9). Incubation with IL-1β or TNFα had no effect on dehydrogenase activity (n=4, $p=0.25$, Figure 3.10).

3.3.5 Influence of systemic lipopolysaccharide *in vivo*

Systemic LPS administration produced a small increase in 11β-reductase activity in aortic rings (n=6, $p=0.045$, Figure 3.11). 11β-Dehydrogenase activity in mouse aortic rings was unaltered by systemic LPS administration (n=3, $p=0.16$, Figure 3.11).

3.3.6 11βHSD activity in aortic homogenates from obese *ob/ob* mice

NADP-dependent 11β-dehydrogenase activity was detected in the homogenates of aortae from both *ob/ob* and lean littermate control mice. There was no significant difference in activity between the two groups (Figure 3.12; n=8-11; $p=0.62$) suggesting that total 11βHSD activity within mouse aorta is not influenced by the obese phenotype.

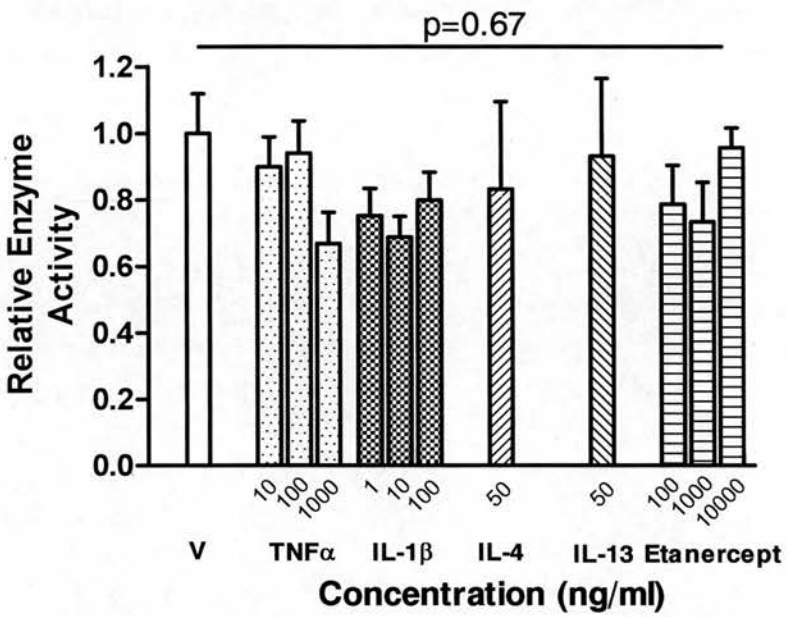

Figure 3.9 Influences of cytokines on 11β-reductase activity in the mouse aorta

Aortic rings were incubated for 48 hours with TNFα, IL-1β, IL-4, IL-13, etanercept or vehicle (V), and then for a further 24 hours in the presence of [^{3}H]-11-dehydrocorticosterone. 11β-Reductase activity is expressed as [^{3}H]-corticosterone formed relative to activity in control incubations without cytokine manipulation. Results are mean ± standard error; there were no differences between groups when compared by single factor ANOVA (p=0.67, n=4-10).

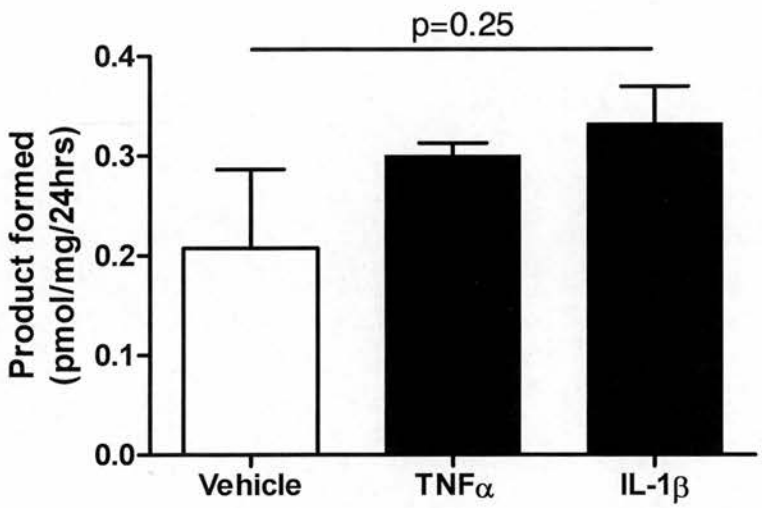

Figure 3.10 Influences of TNFα and IL-1β on 11β-dehydrogenase activity in mouse aorta

11β-Dehydrogenase activity is expressed as the amount of [^{3}H]-11-dehydrocorticosterone formed from [^{3}H]-corticosterone, per milligram (wet weight) over 24 hours in aortic rings from C57B6J mice following incubation with TNFα (100 ng/ml), IL-1β (10 ng/ml) or vehicle control. 11β-Dehydrogenase activity was similar in all groups (p=0.25). Results are mean ± standard error, n=4, and were compared by single factor ANOVA.

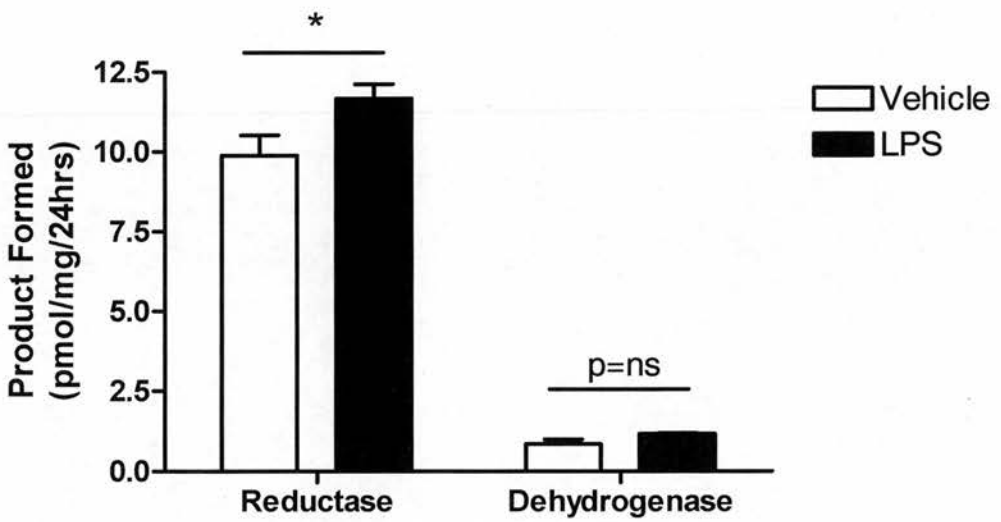

Figure 3.11 Effects of systemic LPS on 11βHSD activity in mouse aorta

11β-Reductase and –dehydrogenase activities are expressed as the amount of [^{3}H]-corticosterone or [^{3}H]-11-dehydrocorticosterone formed, respectively, per milligram (wet weight) over 24 hours in aortic rings from C57B6J mice 6 hours following intraperitoneal administration of lipolysaccharide (10 mg/kg) or vehicle control. 11β-Reductase activity was significantly increased following LPS injection (*p=0.045, n=6) 11β-Dehydrogenase activity was similar in both groups (p=0.16, n=3). Results are mean ± standard error, and were compared by unpaired Student's t-test.

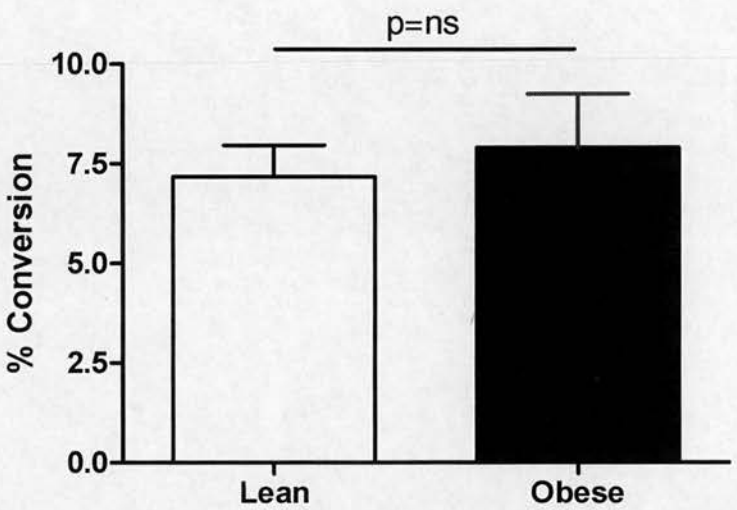

Figure 3.12 11βHSD activity in aortic homogenates from *ob/ob* mice

11βHSD activity is expressed as the percentage conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone (per 5 μg protein) in aortic homogenates from lean and obese mice. 11β-HSD activity was measured after 24 hour incubation in the presence of NAD. 11βHSD activity was not significantly different in the two groups (p=0.62 when compared by unpaired Student's t-test). Results are mean ± standard error, n=8-11.

3.4 Discussion

The intention of these studies was to determine the reaction directionality of the 11βHSD isozymes in intact murine arteries and to investigate the influence of inflammatory mediators on the activity of these enzymes. The results demonstrate the predominant regeneration of glucocorticoids within the vessel wall by 11βHSD1, the sole reductase, and confirm that 11βHSD2 is an exclusive dehydrogenase. In contrast to findings in cell culture, regulation of 11βHSD1 activity by inflammatory mediators does not occur in healthy murine arteries *in vitro*.

11β-Reductase activity in cultured murine aortic SMCs

Initial experiments with cultured murine aortic SMCs confirmed that, consistent with previous studies using human aortic SMCs (Cai *et al.* 2001), 11β-reductase activity was enhanced following stimulation with the inflammatory cytokine IL-1β. However, the effect of IL-1β on 11β-reductase activity was less marked in murine aortic SMCs (40% increase in activity) than the five-fold enhancement shown previously in human SMCs (Cai *et al.* 2001). Moreover, in repeat experiments, both basal 11β-reductase activity within murine SMCs and the response to IL-1β was found to be highly variable, with as much as 100% increase in basal activity and often no measurable up-regulation of this activity following IL-1β stimulation. It is, therefore, possible that cytokine-mediated regulation of 11βHSD1 activity in cultured smooth muscle cells differs between species. The decline in basal 11β-reductase activity with increasing passage raises the possibility that 11βHSD activity may also depend on the degree of cellular proliferation and/or differentiation. This hypothesis is supported by studies which have demonstrated differences in both basal and stimulated 11βHSD1 activity in human ovarian carcinoma cell lines compared with primary cultures of ovarian surface epithelial cells (Gubbay *et al.* 2005). Preliminary studies showed that whilst 11β-reductase activity in MA-SMCs was either unchanged or only modestly up-regulated upon stimulation with IL-1β, co-incubation with pharmacological doses of exogenous corticosterone resulted in a more marked increase in 11β-reductase activity. Although these enzyme activity assays were performed using cells which had been sustained for at least 16 hours in

serum-free conditions, the maintenance cell culture medium contained 10% foetal calf serum (FCS). It is therefore possible that exposure to inconstant amounts of serum-derived glucocorticoid explains the variability in both basal and stimulated 11β-reductase activity which was evident. The synergistic effect of glucocorticoids on cytokine-mediated up-regulation of 11βHSD activity noted in these studies is in keeping with some (Sun & Myatt 2003), but not all (Cooper *et al.* 2001), published data, and raises the possibility that physiological or pathological variations in glucocorticoid availability within vascular tissue may also influence the inflammatory regulation of 11βHSD activity. It should be noted that the dose of corticosterone used in the present study (100μM) was far in excess of physiological circulating glucocorticoid levels. This was due to experimental error, and the dose was intended to be 100nM; however, the positive effect of such a high dose on 11βHSD activity (which would be expected to result in feedback inhibition of reductase activity) suggests that physiological variations in glucocorticoid levels may play an important role in the inflammatory regulation of 11βHSD1 in conditions where the HPA axis might be activated, and future work should seek to address this possibility. The potential influences of these factors on the inflammatory regulation of 11βHSD1 highlight the importance of extending these investigations into intact arteries.

Smooth muscle cells from 11βHSD1 homozygous null (-/-) mice did not grow well in culture. This methodological difficulty is relevant, as abnormal proliferation of vascular smooth muscle cells is an important mechanism in vascular remodelling. Moreover, glucocorticoids play a key role in vascular growth, as exogenous glucocorticoid excess reduces rodent smooth muscle cell proliferation (Reil *et al.* 1999) and inhibits angiogenesis (Small *et al.* 2005). Additionally, we have shown an angiostatic effect of endogenous glucocorticoids, mediated by 11βHSD1, which is of pathological importance as 11βHSD1 null mice exhibit greater angiogenic responses in wounds and in infarcted myocardium (Small *et al.* 2005). However, the effects of alterations in 11βHSD activity, and hence endogenous glucocorticoid availability, on smooth muscle cell proliferation have yet to be explored. It is of interest, then, that contrasting effects of exogenous and endogenous oestrogens on vascular SMC

proliferation have been demonstrated: exogenous oestrogens inhibit human vascular smooth muscle cell proliferation (Morey *et al.* 1997; Somjen *et al.* 1998), whilst in the aromatase knockout (ArKO) mouse model, endogenous oestrogen deficiency reduces vascular smooth muscle cell proliferation and enhances cytokine-mediated apoptosis (Ling *et al.* 2004). There may, therefore, be interesting analogies between oestrogens and glucocorticoids with respect to their effects on vascular smooth muscle cell proliferation.

11βHSD activity and directionality in intact mouse arteries

The studies presented here have demonstrated, for the first time, both 11β-reductase and –dehydrogenase activities in intact murine aorta and hindlimb arteries *in vitro*. Conventional activity assays in homogenised tissue preparations have been limited by lack of co-factor specificity, and have failed to resolve the relative contributions of 11β-reductase and 11β-dehydrogenase activities to the overall control of local glucocorticoid availability. Thus the findings here are important, given the presence of both isozymes in the mouse aorta (Christy *et al.* 2003), and the potential for 11βHSD1 to act in a bi-directional fashion, as both reductase and dehydrogenase (Brem *et al.* 1995; Souness *et al.* 2002). Whilst dehydrogenase activity was similar in the vessels examined, there appear to be regional differences in basal 11β-reductase activity, a finding that has been noted previously (Walker *et al.* 1991), with higher activity in aorta than in the iliofemoral arteries. Interestingly, under these assay conditions, 11β-reductase activity in vascular tissues exceeded that in liver. However, these data should be interpreted with caution as the *in vitro* assay was established to assess 11βHSD activity in viable vascular tissue, and the viability of other tissues over time under these conditions has not been verified. Indeed, the biphasic dehydrogenase activity observed in the liver time course experiment may represent initial 11βHSD1 dehydrogenase activity as a result of liberation of enzyme from the cut surfaces of the liver slice, and then after a few hours, cell death within the rest of the liver slice resulting in further cellular leakage of 11βHSD1 and renewed dehydrogenase activity at later time points.

Experiments using vascular tissue from 11βHSD1-/- mice have confirmed that, in keeping with previous reports, 11βHSD1 acts as the sole reductase (Kotelevtsev *et al.* 1997), and that 11βHSD2 is an exclusive dehydrogenase (Agarwal *et al.* 1994). The persistence of dehydrogenase activity in intact aorta from 11βHSD2 -/- mice *in vitro* is evidence that 11βHSD1 may be acting as both reductase and dehydrogenase under these assay conditions. The question of whether 11βHSD1 has the potential to act in a bi-directional fashion, as both reductase and dehydrogenase, *in vivo* is controversial (Seckl & Walker 2004; Tomlinson *et al.* 2004; Brem *et al.* 1995). The findings from the *in vitro* studies presented here do not provide absolute confirmation of bi-directional activity of 11βHSD1 in intact vascular tissue as the persistent dehydrogenase activity seen in vessels from 11βHSD2-/- mice may be a function of the assay, simply reflecting a change in directionality of 11βHSD1 upon liberation of the enzyme from its intracellular environment, such as occurs in homogenised tissue preparations (Lakshmi & Monder 1988). The novel hypothesis that hexose-6-phosphate dehydrogenase confers reductase activity upon 11βHSD1 in intact cells is intriguing (Hewitt *et al.* 2005). However, although H6PDH expression has been noted in many rat tissues, including the heart (Mandula *et al.* 1970), it has not yet been identified in cells of vascular origin. In any case, despite the potential contribution to total dehydrogenase activity from 11βHSD1, a comparison of the absolute reductase and dehydrogenase activities in intact vascular tissue from wild type mice demonstrates that the reductase direction predominates by approximately 10:1 *in vitro*.

Regulation of 11βHSD activity in intact murine arteries by inflammatory mediators

Extensive examination of the influence of cytokines on 11βHSD activity in intact aortic rings was undertaken. In contrast to the findings in cell culture, there was no evidence of up-regulation of vascular 11βHSD1 reductase activity by cytokines in intact tissue *in vitro*. The pro-inflammatory cytokines IL-1β and TNFα had no effect on 11β-reductase activity, and no counter-regulatory effect on 11β-dehydrogenase activity. Bioactivity of TNFα was confirmed by neutrophil apoptosis assay (Section 2, Figure 2.3) and IL-1β by a positive effect on 11β-reductase activity in cultured cells (Figure 3.1). Furthermore, the anti-inflammatory Th2 cytokines, IL-4 and IL13,

at doses which up-regulate 11βHSD1 activity in human peripheral blood monocytes (Thieringer *et al.* 2001), were also ineffective at regulating 11βHSD1. To investigate the possibility that the presence of endogenous inflammation, and increased TNFα production, within the intact aortic ring was resulting in maximal stimulation of basal 11β-reductase activity, the influence of the TNFα antagonist, etanercept, was examined. The lack of effect of etanercept on 11β-reductase activity suggests that endogenous activation of 11βHSD1 by TNFα does not account for the inability of exogenous TNFα to enhance 11β-reductase activity.

There are a number of studies reporting effects of inflammatory cytokines on 11βHSD1 activity in a variety of different cell types (see Table 1.1). The majority of these demonstrate a selective increase in 11β-reductase activity and/or expression upon stimulation with pro-inflammatory cytokines (Cai *et al.* 2001; Sun & Myatt 2003; Tomlinson *et al.* 2001; Rae *et al.* 2004; Yong *et al.* 2002; Cooper *et al.* 2001; Escher *et al.* 1997; Tetsuka *et al.* 1999). However, this effect is not universal, as TNFα has no effect on 11β-reductase activity in cultured human hepatocytes (Tomlinson *et al.* 2001). Furthermore, in circulating monocytes, 11βHSD1 expression is not up-regulated by the pro-inflammatory cytokines TNFα or IL-1β, but is induced during differentiation into macrophages, and also following exposure to the Th2 cytokines IL-4 and IL-13 (Thieringer *et al.* 2001). All studies reported to date have utilised cell culture systems, which undoubtedly alter the natural cell phenotype. The discrepancy between the ability of cytokines to up-regulate 11βHSD1 activity in cell culture but not in intact tissue preparations suggests that the regulation of 11βHSD1 by inflammatory stimuli may be tissue specific and may also depend on the degree of cell proliferation and/or differentiation.

In contrast to the *in vitro* findings, there was a small (18%) selective increase in 11β-reductase activity in aortic rings of mice which had received *in vivo* LPS. Thus, although individual cytokines appear ineffective at enhancing 11βHSD1 activity in intact tissue, the result of *in vivo* LPS may be to produce an altered inflammatory "milieu" which favours a modest increase in 11β-reductase activity. It is open to

conjecture as to whether the resultant change in glucocorticoid availability following these modest effects could have physiologically relevant consequences.

Finally, total aortic 11βHSD activity in obese *ob/ob* mice, a condition associated with systemic inflammation (Hotamisligil *et al.* 1993), was unaltered compared with lean littermates, supporting the concept that inflammatory regulation of 11βHSDs in tissue or homogenate preparations does not mirror that found in cell culture. It is noteworthy that although alterations in 11βHSD1 have been demonstrated in this murine model of obesity (and also in rats and humans, reviewed by Wake & Walker (2004)) these appear to be tissue-specific, with an increase in 11βHSD1 in liver (Liu *et al.* 2003), and a decrease in adipose (Masuzaki *et al.* 2001; Alberts *et al.* 2003). That there may be discordant changes in 11βHSD1 in different tissues in response to the same systemic disease only serves to highlight the complexity of 11βHSD1 regulation.

Conclusions

In summary, these data confirm the presence, and describe the directionality, of both 11βHSD1 and 11βHSD2 activity in intact murine vascular tissue *in vitro*. Although there was evidence of inflammatory regulation of 11βHSD1 in cultured MA-SMCs, which may depend on cellular phenotype, a similar effect was not evident in healthy intact vascular tissue *in vitro*. In contrast, systemic inflammation *in vivo* produced a modest up-regulation of 11β-reductase activity in intact vascular tissue, suggesting that there may be factors present *in vivo* but not *in vitro* which are required for the inflammatory regulation of 11βHSD1 to be manifest. Although these data do not consistently support the idea that there is inflammatory regulation of 11βHSDs, it should not be inferred that 11βHSD1 does not play a role in modulating glucocorticoid signalling since the current studies demonstrate the predominant regeneration of glucocorticoids within the vessel wall. To explore the inflammatory regulation of vascular 11βHSD activity further, under more physiological conditions, it was felt pertinent to develop a model to allow determination of 11βHSD activity in a regional perfused territory *in situ*.

Chapter Four

Glucocorticoid metabolism in the murine perfused hindquarter

4.1 Introduction

The importance of the 11β-hydroxysteroid dehydrogenases (11βHSDs) in the regulation of local vascular glucocorticoid concentrations, and the contributions of each isozyme to overall 11β-reductase and dehydrogenase activities, have been underlined by the *in vitro* studies described in Chapter 3. Selective regulation of isozyme activity by inflammatory mediators (Cai *et al.* 2001), which was demonstrated in cultured smooth muscle cells, may have a significant influence on glucocorticoid-mediated modulation of vascular function. However, the lack of effect of cytokines on 11βHSD activity within intact vascular tissue *in vitro* (despite up-regulation of 11βHSD1 in response to inflammation *in vivo*) has challenged the notion that these findings can be extrapolated to the *in vivo* environment. Thus, it was deemed important to extend the studies into a more physiologically relevant model of vascular inflammation.

The method for perfusion of the rodent hindquarter (Brandes *et al.* 2000) provides an attractive model for determination of 11βHSD activity and endothelial cell function within a perfused vascular bed. It is a physiological model and has been used to explore both metabolism (Ye & Colquhoun 1998a; Ohshima *et al.* 1989) and vascular function (Emeis 1983; Brandes *et al.* 2000; Tranquille & Emeis 1990; Ceiler *et al.* 1999; McAllister 2003; Bohlooly *et al.* 2001) in an intact vascular territory. It was selected in preference to other methods (for example, the perfused mesenteric bed) as it allows both perfusion of large and small vessels and measurement of biochemical signals within a physiological environment. Although metabolism within the perfused hindquarter has been studied in detail (Ye & Colquhoun 1998a; Ohshima *et al.* 1989), glucocorticoid metabolism by the 11βHSDs has not been investigated previously.

The studies presented in this chapter explore the hypothesis that systemic inflammation up-regulates 11βHSD1 reductase activity in the perfused mouse hindquarter. The specific aims of the investigations were (1) to develop a murine hindquarter perfusion model, (2) to characterise the activity and reaction

directionality of the 11βHSDs within this regional perfused territory, and (3) to assess the influence of systemic inflammation on 11βHSD1 activity. The studies required the application of existing techniques of hindquarter perfusion and of systemic inflammation. It was also necessary to develop a novel method with which to determine the activities of 11βHSD1 and 11βHSD2 in the murine perfused hindquarter.

4.2 Methods

4.2.1 Mice

Unless otherwise specified, all hindquarter perfusions were performed using male C57B6J mice aged 8-16 weeks. Mice were killed by cervical dislocation prior to commencement of perfusion studies, as anaesthetic agents are known to have effects on mediators of vascular tone (Toda *et al.* 1992; Zuo *et al.* 1996).

4.2.2 Method Development

A model of mouse hindlimb perfusion was required which would allow infusion of radiolabelled steroids, collection of perfusate for analysis and determination of perfusion pressure. This would provide a relatively physiological model in which to determine the significance of glucocorticoid metabolism by 11βHSDs. Therefore, a method was developed (see Chapter 2, Section 2.9) based on studies by Brandes *et al.* (2000) to allow selective perfusion of the hindquarters, with physiological buffer containing steroids, via a cannula sited in the distal aorta. 11βHSD activity was determined in this model by collecting aliquots of venous effluent, over the course of the perfusion study, from a cannula sited in the distal inferior vena cava. Analysis of the proportion of radiolabelled 11-dehydrocorticosterone and corticosterone within each aliquot by HPLC (as described in Methods Section 2.10) allowed determination of enzyme activity. Conditions were optimised during pilot studies using a variety of flow rates, buffer compositions, temperatures and surgical techniques, and these are detailed in the following sections. Attempts to study vascular function within this

hindquarter model proved difficult. Few publications have reported the use of a mouse hindlimb model for functional studies (Brandes *et al.* 2000; Bohlooly *et al.* 2001), and some of these have clearly been hampered by methodological difficulties (Bohlooly *et al.* 2001). Moreover, the purposes of these particular functional studies in the context of this thesis were superseded by findings in parallel human studies, detailed in Chapter 5.

4.2.2.1 Perfusion Set Up

The aim of the surgical set up described in Chapter 2 (Section 2.9.1 and Figure 2.1) was to allow selective perfusion of both hindlimbs, without retrograde (or otherwise) perfusion of metabolically active abdominal or pelvic viscera. The perfused region was confirmed in preliminary perfusion studies using C57B6J mice (n=3) in which KH buffer containing Evans Blue dye (0.5% w/v) was used to evaluate the perfused regions (Hamza & Kaufman 2004).

4.2.2.2 Perfusion Conditions

Initial perfusion experiments used modified Krebs-Henseleit (KH) buffer, based on studies by Brandes *et al.* (2000). However perfusion of hindquarters with this buffer resulted in significant tissue oedema, which could be prevented with the addition of 2% bovine serum albumin (Ye & Colquhoun 1998b; Bohlooly *et al.* 2001). Thus, all perfusion studies described in this chapter have utilised a modified KH buffer containing 2% bovine serum albumin.

Animals were kept on a warmed dish at 37°C and perfused with buffer at the same temperature (Bohlooly *et al.* 2001). To achieve a buffer temperature of 37°C at the abdominal aorta, preliminary studies indicated that the buffer required to be maintained in a water bath at 40°C.

A constant-flow approach, using a peristaltic pump, was used to perfuse the hindquarters. Flow rates of 0.8-1.2 ml/min were chosen to approximate physiological

perfusion pressures (~40 mmHg) within the maximally-dilated hindlimb and to avoid tissue oedema. Inflow was determined by timed collection of venous effluent, and perfusion pressure was monitored during initial flow vs. pressure perfusion studies using a transducer connected to a side port of the perfusion system (data not shown). The pressure transducer was immediately proximal to the aortic cannula and at the same level as the animal, and was connected to a Powerlab analogue-to-digital converter.

Standard conditions for the perfusion studies utilised buffer containing [^{3}H]-corticosterone or [^{3}H]-11-dehydrocorticosterone at concentrations of 5 nM. These concentrations were chosen to be sufficient for detection by HPLC and were close to physiological levels (circulating corticosterone and 11-dehydrocorticosterone concentrations in the mouse vary diurnally within the ranges 25-150 nM and 0-10 nM, respectively (Harris *et al.* 2001)).

4.2.2.3 Steroid Recovery

In all perfusion studies, 20 μl aliquots of effluent samples and perfusate (stock buffer which had not been perfused through the hindquarter) were added to 3 mls Zinsser Aquasafe 300 plus scintillation fluid and analysed by scintillation counting (Packard Tri-carb 2100TR) to estimate steroid content. The proportion of steroid recovered in the effluent was then calculated from the counts per minute (c.p.m.) in each aliquot as:

% Recovery = (c.p.m. recovered in effluent / c.p.m. recovered in perfusate) x 100

4.2.3 Glucocorticoid metabolism in the perfused hindquarter

4.2.3.1 11β-Reductase activity

11β-Reductase activity was determined in perfused hindquarters of wild type mice (n=3) using standard conditions (as described in Section 2.9), over a 55 minute

period. Buffer contained 5 nM [^{3}H]-11-dehydrocorticosterone and aliquots of effluent were collected over 5 minute intervals throughout the perfusion.

In further studies, reductase enzyme kinetics were established by perfusing hindquarters over 60 minutes under similar conditions, except with varying concentrations of [^{3}H]-11-dehydrocorticosterone (5, 7.5, 15, 30 and 500 nM; n=3 animals at each concentration). Aliquots of effluent were collected over 90 seconds at intervals throughout the study.

4.2.3.2 11β-Reductase activity in 11βHSD1 -/- mice

11β-Reductase activity was determined in the perfused hindquarters of 11βHSD1-/- and age-matched C57B6J mice (n=3) as described above (Section 4.2.3.1).

4.2.3.3 11β-Dehydrogenase activity

11β-Dehydrogenase activity was determined (as described in Section 2.9) in the perfused hindquarters of wild type mice (n=4) over a 55 minute time period. Buffer contained 5 nM [^{3}H]-corticosterone and aliquots of effluent were collected over 5 minute intervals throughout the perfusion.

4.2.3.4 11β-Dehydrogenase activity in 11βHSD2 -/- mice

11β-Dehydrogenase activity was determined in the perfused hindquarters of male 11βHSD2-/- and age-matched C57B6J mice (n=3-4) over a 55 minute time period as described above (Section 4.2.3.3).

4.2.4 11β-Reductase activity in hindlimb tissues *in vitro*

In order to establish which tissues within the hindlimb were contributing to the observed 11β-reductase activity, iliofemoral arteries, pieces of quadriceps muscle, and skin and subcutaneous adipose tissue were dissected from C57B6J mice and

assayed for 11β-reductase activity *in vitro* using the method described for arteries in Section 2.8.3 (n=4).

4.2.5 Effect of LPS on 11β-reductase activity

To observe the effects of systemic inflammation on 11β-reductase activity in the perfused hindquarter, perfusion studies were performed on mice which had received intraperitoneal LPS (10 mg/kg) or vehicle 6 hours previously (as described in Section 2.9.4). Perfusions lasted 60 minutes, and aliquots were collected over 90 second intervals throughout the study.

4.2.6 Statistics

Data are expressed as mean ± standard error and were analysed by Student's t-test or ANOVA as appropriate.

4.3 Results

4.3.1 Method Development

4.3.1.1 Perfusion Set Up

Use of Evans blue dye confirmed that there was no retrograde perfusion of abdominal viscera or of the testes, epidiymis or epididymal adipose tissue. Evans Blue staining was seen in the hindlimbs, the tail, the inferior peritoneal lining and the *glandula vesicularis*, suggesting that the perfused territory was not confined to the hindlimbs alone, but rather encompassed perfusion of bilateral hindquarters.

4.3.1.2 Steroid Recovery

During perfusion of C57B6J hindquarters with [^{3}H]-11-dehydrocorticosterone (5-500 nM, n=15), the recovery of perfused steroid increased linearly to reach a steady state

within approximately 15 minutes. Once this equilibrium was reached, approximately 90% of radiolabelled steroid was recovered (Figure 4.1). Similar recoveries were calculated for studies infusing [^{3}H]-corticosterone, and also in studies with 11βHSD1-/- or 11βHSD2-/- mice (data not shown).

4.3.2 Glucocorticoid Metabolism in the Perfused Hindlimb

4.3.2.1 11β-Reductase Activity

11β-Reductase activity was detected in the perfused hindquarters of C57B6J mice, as indicated by conversion of [^{3}H]-11-dehydrocorticosterone to [^{3}H]-corticosterone (Figure 4.2). 11β-reductase activity increased throughout the study to reach a maximum of approximately 80% conversion (equivalent to a velocity of 3.2 pmol [^{3}H]-corticosterone / minute).

4.3.2.2 11β-Reductase Kinetics

11β-Reductase activity increased in proportion to substrate concentration (Figure 4.3). Kinetic constants for 11β-reductase were calculated from the Lineweaver-Burke linear transformation of the Michaelis-Menten equation (Lineweaver & Burk 1934), using the mean data (n=3) of enzyme activity at the 11 minute time point (which is midway along the linear initial phase of product generation, and thus approximates to initial reaction velocity, Figure 4.4). These data gave an apparent K_m of 1.064 μM and a maximum velocity 313 pmol/min.

4.3.2.3 11β-Reductase in 11βHSD1 -/- Mice

In contrast to studies using wild type mice, no 11β-reductase activity was detected in hindquarters of 11βHSD1-/- mice (Figure 4.2).

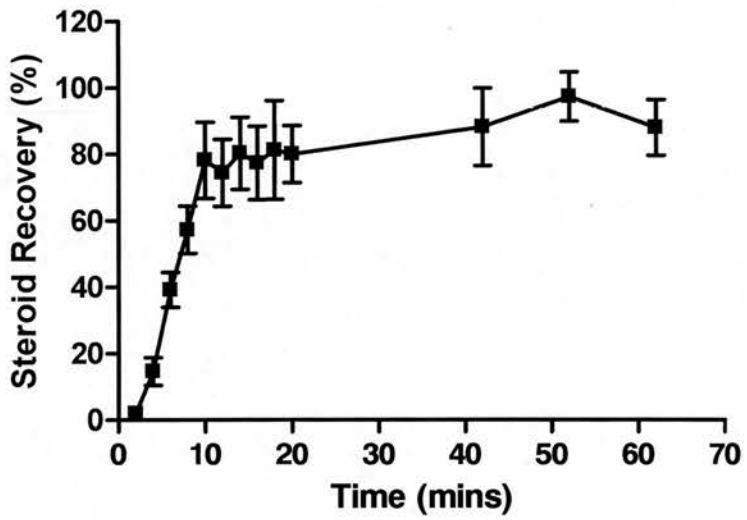

Figure 4.1 Steroid recovery following hindquarter perfusion of C57B6J mice

Percentage of total [^{3}H]-steroid recovered from effluent during perfusion of C57B6J hindquarters with 5-500 nM [^{3}H]-11-dehydrocorticosterone. [^{3}H]-steroid content was estimated by scintillation counting of 20 μl aliquots of perfusate (stock buffer which had not been perfused through hindquarters) and effluent samples. % Recovery was calculated as (c.p.m. recovered in effluent / c.p.m. recovered in perfusate) x 100. Results are mean ± standard error; n=15.

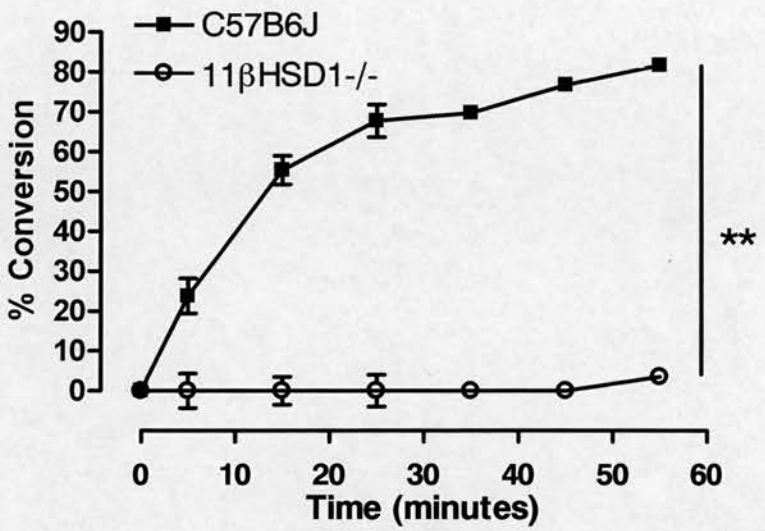

Figure 4.2 11β-Reductase activity in perfused hindquarters of C57B6J and 11βHSD1-/- mice

11β-Reductase activity in perfused hindquarters of C57B6J (closed squares) and 11βHSD1-/- (open circles) mice. Enzyme activity is expressed as the percentage of [^{3}H]-corticosterone formed from [^{3}H]-11-dehydrocorticosterone. 11β-Reductase activity was abolished in 11βHSD1-/- mice. Results are mean ± standard error; n=3, **p<0.005, compared with controls.

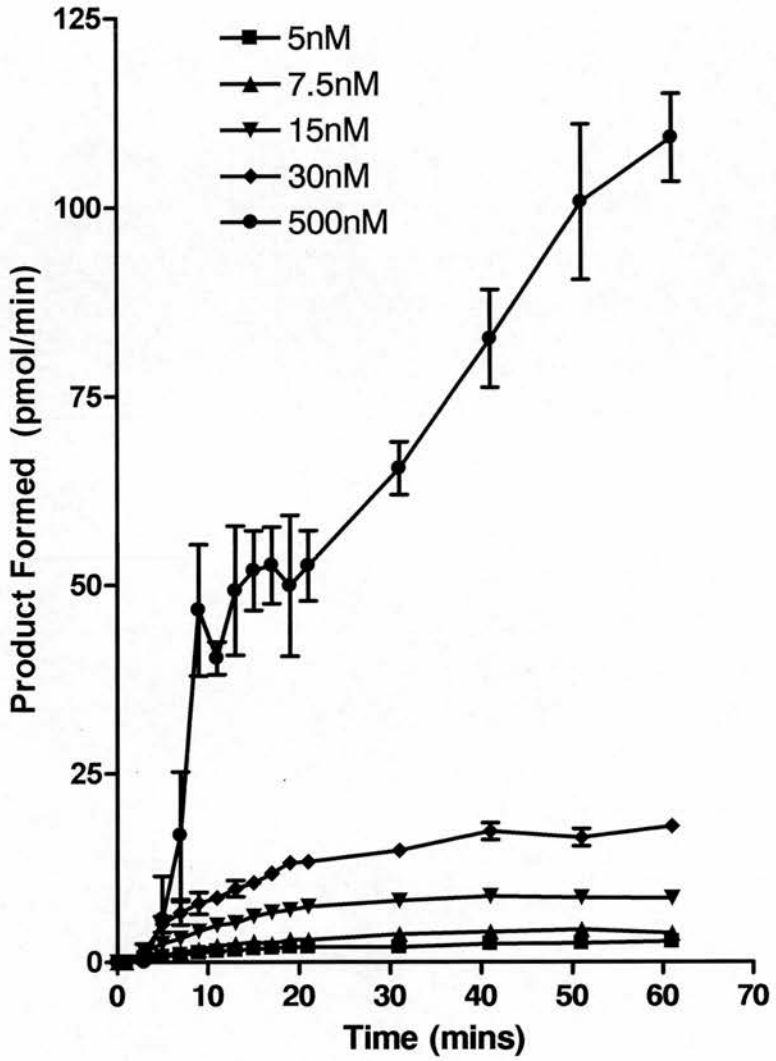

Figure 4.3 Effect of substrate concentration on 11β-reductase activity in the perfused hindquarter

Effect of increasing substrate concentration (5-500 nM 11-dehydrocorticosterone) on enzyme activity in the perfused hindquarter. C57B6J mice underwent 60 minute hindquarter perfusions. Results are mean ± standard error, n=3 at each concentration.

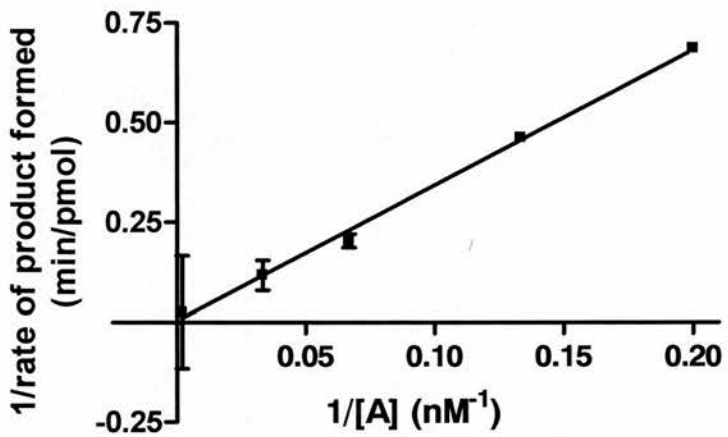

Figure 4.4 11β-Reductase kinetics in the perfused hindquarter

Lineweaver-Burke plot showing kinetics of 11β-reductase activity in perfused hindquarters of C57B6J mice. Using a time point of 11 minutes following the commencement of the perfusion, K_m was calculated as 1.064 μM and maximum velocity (V_{max}) 313 pmol/min. Results are mean ± standard error, n=3 at each concentration. A = 11-dehydrocorticosterone.

4.3.2.4 11β-Dehydrogenase Activity

11β-Dehydrogenase activity, albeit at a very low level, was detected in the perfused hindquarters of C57B6J mice, as indicated by conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone (Figure 4.5). In keeping with previous *in vitro* findings, dehydrogenase activity in hindquarters of wild type mice (approximately 6% conversion, or 0.24 pmol [^{3}H]-11-dehydrocorticosterone/minute) was approximately 10-fold lower than reductase activity (80% conversion, or 3.2 pmol [^{3}H]-corticosterone/minute).

4.3.2.5 11β-Dehydrogenase activity in 11βHSD2 -/- Mice

In contrast with the findings *in vitro* (Chapter 3), dehydrogenase activity was below the limit of detection by HPLC (2% conversion) in hindquarters of mice with genetic inactivation of 11βHSD2 (Figure 4.5).

4.3.3 11β-Reductase activity in hindlimb tissues *ex vivo*

Ex vivo activity assays of tissues from the hindlimb confirmed the presence of reductase activity in the hindlimb vasculature (11.4±31.4 pmol/mg/24hrs), skeletal muscle (0.14±0.02 pmol/mg/24hrs) and skin and subcutaneous adipose tissues (0.27±0.05 pmol/mg/24hrs). Weight for weight, reductase activity in the vasculature far exceeded that in the skeletal muscle or skin/subcutaneous adipose ($p<0.0001$, n=4, Figure 4.6).

4.3.4 Effects of LPS on 11β-reductase activity in the perfused hindquarter

Lipopolysaccharide administration resulted in significant weight loss ($p<0.05$, n=6) and splenomegaly ($p<0.05$, n=6) compared with vehicle injection (Figure 4.7). The proportion of steroid recovered in effluent from perfused hindquarters was not influenced by LPS treatment ($p>0.05$, n=6, Figure 4.8).

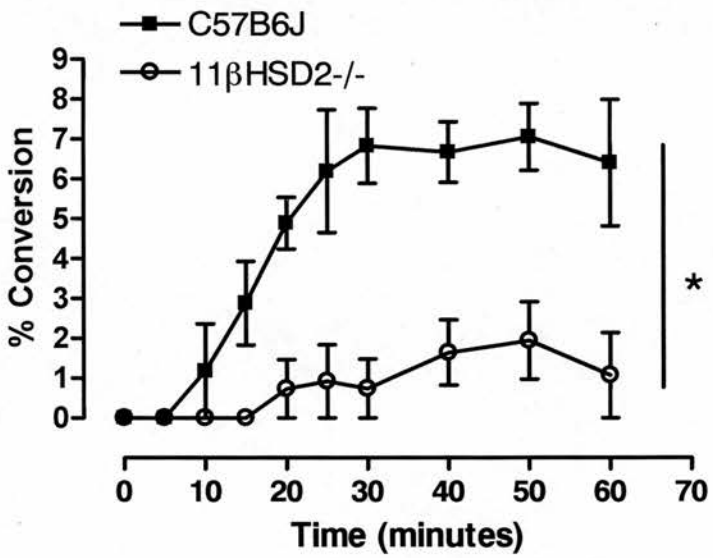

Figure 4.5 11β-Dehydrogenase activity in perfused hindquarters of wild type and 11βHSD2-/- mice

11β-Dehydrogenase activity in perfused hindquarters of C57B6J (closed squares) and 11βHSD2-/- (open circles) mice. Enzyme activity is expressed as the percentage of [^{3}H]-11-dehydrocorticosterone formed from [^{3}H]-corticosterone. 11β-Dehydrogenase activity in 11βHSD2-/- mice was below the limit of detection by HPLC (conversion all less than 2%). Results are mean ± standard error; n=3-4, *$p<0.05$, compared with controls. Note difference in Y-axis range when making comparison with Figure 4.2.

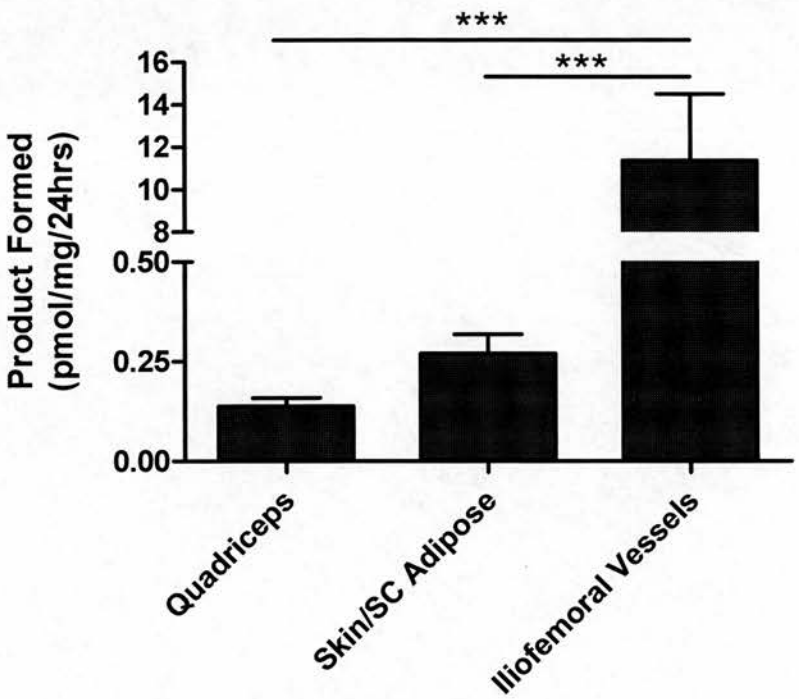

Figure 4.6 11β-Reductase activity in hindlimb tissues

11β-Reductase activity is expressed as the amount of [^{3}H]-corticosterone formed from [^{3}H]-11-dehydrocorticosterone, per milligram (wet weight) over 24 hours, in intact iliofemoral vessels, chunks of quadriceps muscle and skin and subcutaneous adipose tissue from C57B6J mice (n=4). 11β-Reductase activity in ileofemoral vessels far exceeded that in the skeletal muscle and skin and subcutaneous adipose (***$p<0.0001$). Results are mean ± standard error, n=4.

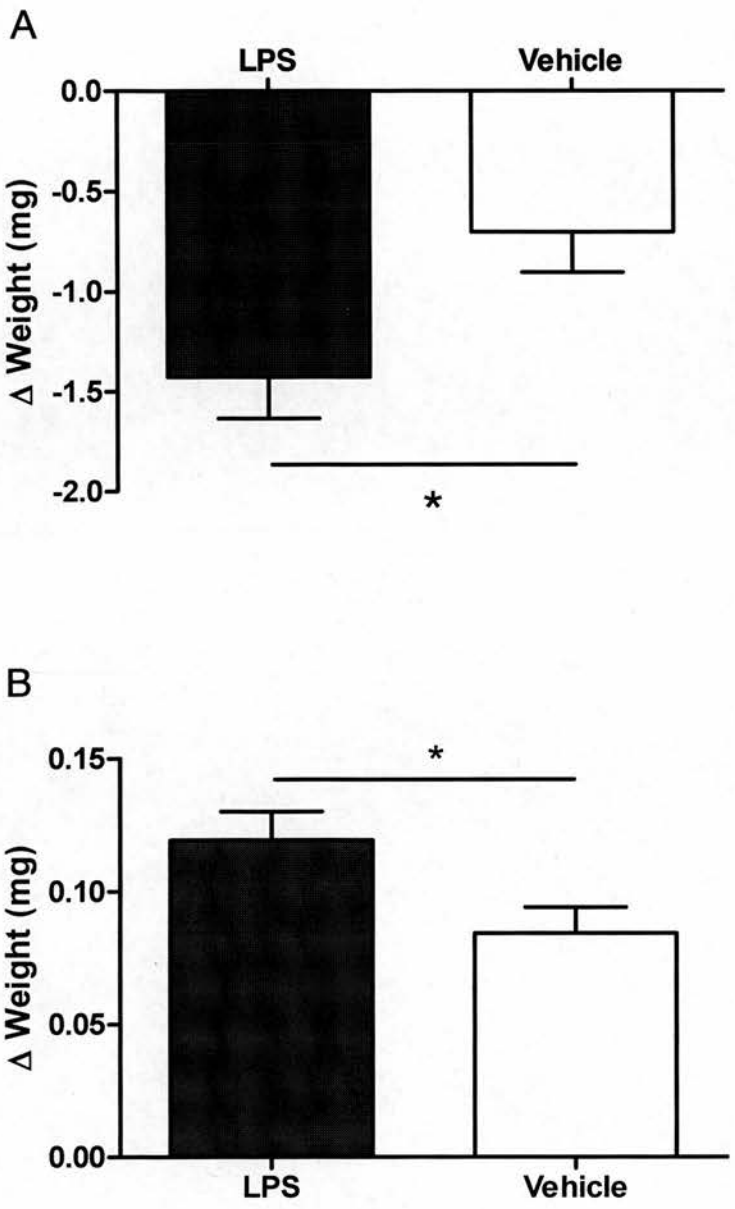

Figure 4.7 Effects of LPS on body weights and spleen weights

Changes in (A) total body weight and (B) spleen weights of C57B6J mice, 6 hours following intraperitoneal LPS (10 mg/kg, solid bars) or saline vehicle (open bars). LPS administration resulted in significant weight loss (*$p<0.05$) and splenomegaly (*$p<0.05$) compared with vehicle. Results are mean ± standard error, n=10.

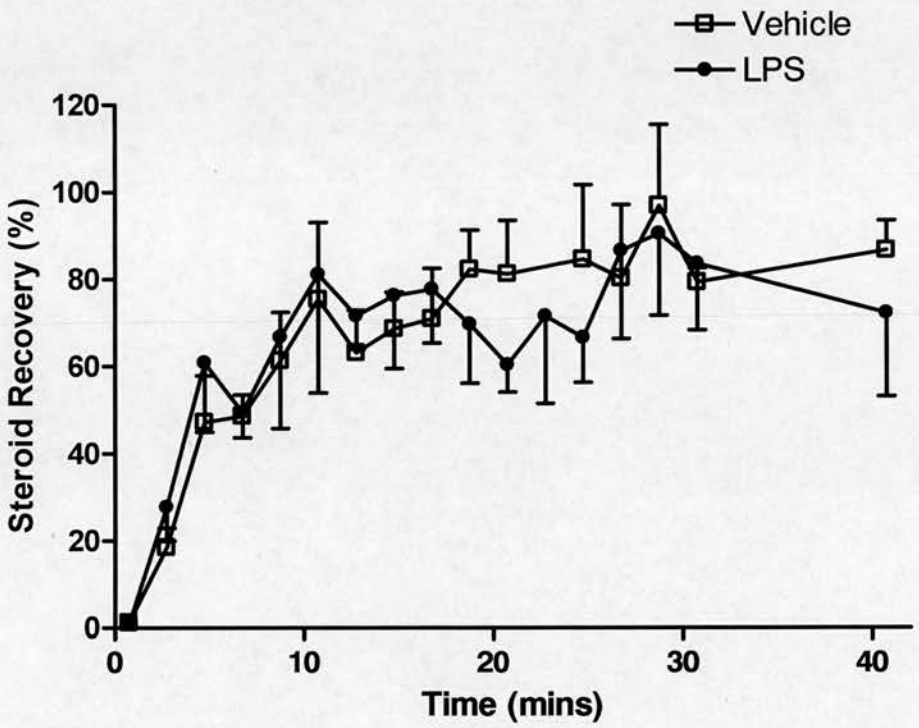

Figure 4.8 Effects of LPS on total steroid recovery

Percentage of total [^{3}H]-steroid recovered from the effluent of C57B6J hindquarters perfused with 5nM [^{3}H]-11-dehydrocorticosterone. Mice had received either LPS (10 mg/kg, closed circles) or saline (20 ml/kg, open circles) intraperitoneally 6 hours previously. [^{3}H]-steroid content was estimated by scintillation counting of 20 μl aliquots of perfusate (stock buffer which had not been perfused through an animal) and effluent samples. % Recovery was calculated as (c.p.m. recovered in effluent / c.p.m. recovered in perfusate) x 100. Results are mean ± standard error, n=6.

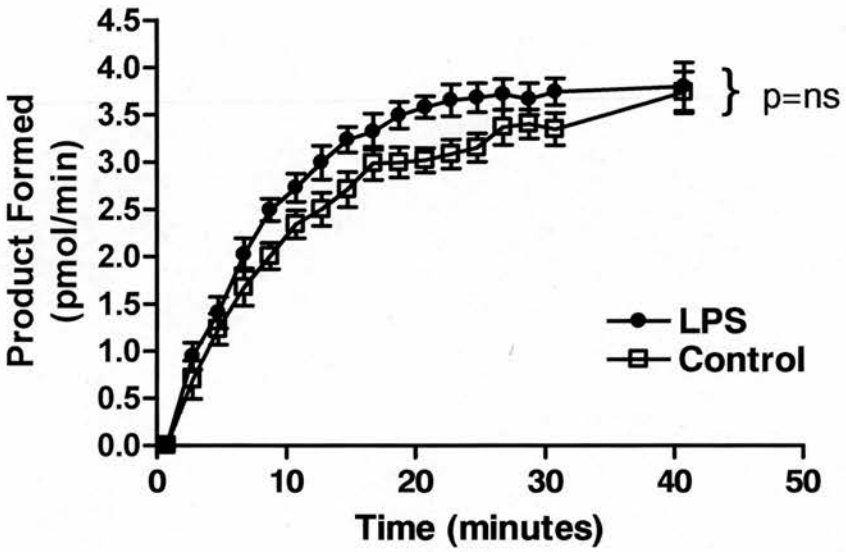

Figure 4.9 Effects of LPS on 11β-reductase activity in the perfused hindquarter

11β-Reductase activity in perfused hindquarters of C57B6J mice 6 hours following *in vivo* intraperitoneal administration of lipolysaccharide (LPS, 10 mg/kg, closed circles) or vehicle (open squares). 11β-Reductase activity is expressed as the amount of [^{3}H]-corticosterone formed. Results are mean ± standard error; there were no differences between groups (p=0.12 by repeated measures ANOVA, n=6).

11β-Reductase activity in the perfused hindquarter was similar in animals treated with LPS compared with vehicle controls, although a trend to a modest increase in activity following LPS treatment was noted (p=0.12, n=6, Figure 4.9).

4.4 Discussion

The intention of these studies was to determine the activity and reaction directionality of the 11βHSD isozymes in the perfused mouse hindquarter, and to investigate the influence of systemic inflammation on the activity of these enzymes. The results demonstrate the presence of both 11β-reductase and –dehydrogenase activities in the perfused hindquarter, with 11β-reduction being the predominant reaction direction, consistent with the *in vitro* findings from Chapter 3. Again in keeping with results in Chapter 3, 11βHSD2 was confirmed as an exclusive dehydrogenase. However, whereas 11βHSD1 may have bidirectional activity in arteries *in vitro*, it appeared to act solely as a reductase in the perfused hindquarter *in situ*. Whilst systemic LPS had a modest effect on 11β-reductase activity in aortic rings *ex vivo*, this effect was not evident in perfused hindquarters suggesting that up-regulation of 11βHSD1 reductase by inflammatory stimuli is unlikely to be an important accompaniment of vascular inflammation *in vivo*.

11βHSD activity and directionality in the perfused hindquarter

These are the first studies to determine the presence of 11βHSD activity in a regional vascular bed *in situ*. The data from the studies described in this chapter suggest that the hindquarter model is a robust, reproducible technique with which alterations in 11βHSD activity can be investigated. Both 11β-reductase and 11β-dehydrogenase activities were detected. In keeping with the *in vitro* vascular studies described in the previous chapter, the reductase direction predominated by ~10-fold, illustrating again the predominant regeneration of glucocorticoids within a vascular territory.

Reductase activity within the hindlimb appears substantial, particularly in relation to the lower dehydrogenase activity, with around 65% of 11-dehydrocorticosterone being converted to corticosterone after just 30 minutes. However, the absolute magnitude of this enzyme activity represents a reaction velocity of approximately 3 pmol of corticosterone produced per minute. This level of activity is significantly lower (by around 20-fold) than that found in rat livers perfused under similar conditions, where the enzyme activity is of the order of 70 pmol of corticosterone generated per minute (Jamieson *et al.* 2000). Considering 11βHSD1 is highly expressed in the liver, a key site for glucocorticoid regeneration, it is perhaps not surprising to find much lower levels of 11β-reductase activity in the hindlimb. Interestingly, in contrast to the results from the murine studies presented here, investigations using the deuterated cortisol tracer method (Andrew *et al.* 2002) to measure 11βHSD activities in humans *in vivo* failed to detect generation of active glucocorticoid within the leg (Basu *et al.* 2004). These apparent differences between enzyme activities in human leg and murine hindlimb may highlight species-specific variations in 11βHSD1 (Ricketts *et al.* 1998a), or may simply reflect differences between the investigative methods employed.

More detailed kinetic studies of 11β-reductase activity revealed an apparent K_m of ~1 μM in the perfused hindlimb, consistent with values reported for 11β-reduction by 11βHSD1 *in vitro* (Agarwal *et al.* 1990) (Pu & Yang 2000; Shafqat *et al.* 2003), and is evidence that the reductase activity present in the hindlimb can be attributed to the 11βHSD1 isozyme. This K_m value suggests that the affinity of 11βHSD1 for corticosterone within the hindquarter is similar to that found *in vitro*. There is an enigma in this, however, since endogenous concentrations of 11-dehydrocorticosterone, the substrate for 11βHSD1 reduction, are in the low nanomolar range (Harris *et al.* 2001). It is of interest, then, that 11βHSD1 is now known to occur as a dimeric enzyme (Zhang *et al.* 2005a) which appears to obey Michaelis-Menten kinetics for 11β-dehydrogenation but exhibits cooperative kinetics for 11-oxoreduction (Maser *et al.* 2002). This positive cooperativity, in addition to the upregulation of 11βHSD1 by glucocorticoids (Takeda *et al.* 1994c) (Sun & Myatt 2003; Hammami & Siiteri 1991; Jamieson *et al.* 1995; Liu *et al.* 1996), might

provide a flexible system whereby dynamic adaptation in response to wide fluctuations in endogenous glucocorticoid levels is possible.

Perfusion studies using 11βHSD1-/- mice, in keeping with results from *in vitro* studies, confirmed that 11βHSD1 is the sole reductase (Kotelevtsev *et al.* 1997), and that 11βHSD2 is an exclusive dehydrogenase (Agarwal *et al.* 1994). Further experiments, with perfused hindquarters of 11βHSD2 -/- mice demonstrated virtual abolition of 11β-dehydrogenase activity which suggests that, although 11βHSD1 has capacity to act as both reductase and dehydrogenase in arteries *in vitro*, this is likely to be an artefact of the assay (as discussed in Section 3.4) as 11β-reduction is the predominant reaction direction *in vivo*.

The high level of 11βHSD1 reductase activity demonstrated in these perfusion studies raised the question of where, within the hindlimb, 11β-reduction of 11-dehydrocorticosterone was occurring. 11βHSD1 activity has been demonstrated in several cells/tissues which comprise the hindlimb, including subcutaneous adipose (Bujalska *et al.* 1997; Sandeep *et al.* 2005), skeletal myoblasts (Whorwood *et al.* 2001) and, of course, vascular tissue (Christy *et al.* 2003; Brem *et al.* 1998; Brem *et al.* 1995). *Ex vivo* studies described in this chapter demonstrated that hindlimb arteries, skeletal muscle and skin and subcutaneous adipose all have 11β-reductase capacity. Whilst vascular tissue was found to have much higher 11βHSD1 activity than skeletal muscle or skin and subcutaneous adipose tissue weight for weight, the proportional contribution of each tissue to overall glucocorticoid regulation within the hindlimb has not been evaluated. It is therefore likely that all of these tissues make a contribution to the reductase activity determined during perfusion studies. Whilst it could be argued that the hindquarter model is not a "clean" vascular preparation, it may in fact be a more physiologically relevant model of *in vivo* glucocorticoid metabolism, as there may be vascular consequences of altered glucocorticoid levels within adjacent tissues which may be overlooked in isolated vascular preparations.

Effects of LPS on 11βHSD activity the perfused hindquarter

LPS had no significant effect on 11βHSD1 activity in the perfused hindquarter. This is in contrast to *in vitro* findings in which there was a modest increase in 11β-reductase activity in aortic rings of mice which had received *in vivo* LPS. Although the regulatory effect of LPS on 11βHSD1 was not evident in perfused hindlimbs as it was in aortic rings, there was a similar trend towards an increase in 11β-reductase activity, raising the possibility that there is a small effect of LPS on hindlimb vascular 11βHSD1 activity which may be masked by the contributions from other tissue types within the regional perfused territory. Additionally, it is worth considering that regional differences in the inflammatory regulation of vascular 11βHSD1 activity may account for the differing effects of LPS in the hindquarter and the aorta.

Systemic lipopolysaccharide, a well validated model of inflammation, was selected for use in these studies as it is associated with activation of the innate immune system (Parrillo 1993) and release of the pro-inflammatory cytokines TNFα and IL-1β (Corbacho *et al.* 2004; Saito *et al.* 2003). Circulating levels of these cytokines peak within 2-6 hours following LPS injection (Corbacho *et al.* 2004; Saito *et al.* 2003; Sallenave *et al.* 2003). When administered at a dose of 10mg/kg, animals exhibited significant weight loss (in keeping with previous studies (Simon *et al.* 2004)) and splenomegaly, indicative of systemic illness and activation of the immune system. Indeed, mortality studies suggest that this dose produces profound endotoxaemia with mortality rates of 75% at 48 hours (Hirschfield *et al.* 2003). There is no doubt, therefore, that this model of inflammation produces a severe inflammatory phenotype with a rapid onset, and could be predicted to result in up-regulation of vascular 11βHSD1. Although no effect of LPS on hindlimb 11βHSD1 activity was seen, it is possible that there is a positive effect of LPS on 11βHSD1 which is simply not apparent at 6 hours. However, the modest up-regulation of aortic 11βHSD1 activity *ex vivo*, following just such an insult (Chapter 3), suggests that the hindlimb findings are reliable, rather than artefactual, and imply that inflammatory regulation of 11βHSD1 in healthy vessels is absent *in vivo*.

In summary, these data confirm the presence, and describe the directionality, of both 11βHSD1 and 11βHSD2 activity in the perfused mouse hindquarter *in situ*. Whilst systemic inflammation *in vivo* produced a modest up-regulation of 11β-reductase activity in intact vascular tissue (Chapter 3), a similar effect was not seen in the perfused hindquarter suggesting that up-regulation of 11βHSD1 reductase is unlikely to be a significant accompaniment of vascular inflammation in healthy arteries *in vivo*. However, it remains possible that 11βHSD1 may be up-regulated in pathological conditions associated with intense cell proliferation, such as vessel injury or atheroma, or in the context of augmented glucocorticoid availability (e.g., stress, acute illness). It is exciting to speculate further, therefore, that up-regulation of 11β-reductase activity by inflammatory mediators may not be relevant under physiological conditions but may become important during pathophysiological disease processes. To address the hypothesis that 11βHSD1 activity may be regulated by inflammatory mediators under pathological conditions, further studies are required to establish the role of 11βHSD1 in the context of vascular disease states. The hindquarter model described in this chapter will now provide an extremely useful tool for the investigation of 11βHSD1 activity under pathological conditions such as femoral artery injury.

Chapter Five

Effects of acute variations in glucocorticoid availability on endothelial cell function *in vivo*

5.1 Introduction

Systemic glucocorticoid excess is associated with an increased incidence of adverse cardiovascular events (Wei *et al.* 2004; Souverein *et al.* 2004). Indeed, even variations in endogenous glucocorticoid levels within the normal physiological range correlate with long-term cardiovascular risk factors such as hypertension and insulin resistance (Walker *et al.* 1998; Fraser *et al.* 1999; Walker *et al.* 2000). The link between elevated glucocorticoid concentrations and cardiovascular risk may be due, at least in part, to direct changes in vascular reactivity. Glucocorticoids potentiate vasoconstrictor responses to noradrenaline and angiotensin II (Ullian 1999; Walker & Williams 1992), and impair endothelium-dependent vasodilatation (Walker *et al.* 1995b; Mangos *et al.* 2000; Iuchi *et al.* 2003). The mechanism of impaired cholinergic dilatation following glucocorticoid therapy is likely to involve abnormalities of the endothelial nitric oxide system (Hadoke *et al.* 2001; Mangos *et al.* 2000). Glucocorticoid excess, either endogenous or exogenous, is also associated with elevated plasma concentrations of plasminogen activator inhibitor type 1 (PAI-1), and a hypercoagulable state (Sartori *et al.* 2000; Fatti *et al.* 2000; Patrassi *et al.* 1985; Ikkala *et al.* 1985; Patrassi *et al.* 1992; Sartori *et al.* 1999). Within the vessel wall, it is likely that there is a balance between the adverse (inhibition of endothelial nitric oxide generation, altered fibrinolytic and coagulation factors) and beneficial (inhibition of inflammation, attenuation of thrombin-induced mitogenesis) effects of glucocorticoids. Studies described in this thesis have addressed the possibility that inflammation alters 11βHSD activity and hence local glucocorticoid action within vascular tissue. However, it is not known whether acute variations in endogenous glucocorticoid levels, such as those which might occur following alterations in 11βHSD activity, are influential in the balance of these local glucocorticoid effects.

In order to determine whether acute alterations in glucocorticoid availability impair endothelial cell function, it was desirable to use a technique which would allow measurement of endothelial cell function within a physiologically relevant system. Vasodilatation and fibrinolytic capacity are markers of endothelial function, and it is possible to measure these variables *in vivo* in man. The measurement of bilateral

forearm blood flow, by venous occlusion plethysmography, coupled with unilateral brachial artery infusion of vasoactive drugs at sub-systemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses in man *in vivo* (Benjamin *et al.* 1995; Webb 1995). Combined with bilateral forearm venous sampling, this technique also permits the assessment of local release of tissue and endothelium-derived factors (Plumpton *et al.* 1995), including t-PA and PAI-1 (Newby *et al.* 1997; Newby *et al.* 1998; Jern *et al.* 1994b; Jern *et al.* 1994a; Jern *et al.* 1997). The use of this method in healthy volunteers, in conjunction with manipulation of circulating glucocorticoid levels, should allow comparison of the endothelial effects of basal 'physiological' and moderately 'stressed' levels of glucocorticoids with glucocorticoid deficiency.

The study described in this chapter explores the hypothesis that acute variations in circulating glucocorticoid levels (within the physiological range) impair endothelial cell vasomotor and fibrinolytic function. The specific aims of the study were to examine the effects of altered circulating glucocorticoid levels (1) on endothelium-dependent vasodilatation and (2) on basal and stimulated release of t-PA and PAI-1.

5.2 Methods

5.2.1 Subjects

Twelve healthy non-smoking men aged between 18 and 60 years were enrolled in the study which was undertaken with the approval of the Lothian Research Ethics Committee and the written informed consent of each subject. Subjects were excluded if they had biochemical evidence of elevated fasting glucose, or significant hepatic, renal or thyroid dysfunction at an initial screening visit.

5.2.2 Study design

This study compared basal 'physiological' and moderately 'stressed' levels of glucocorticoids with glucocorticoid depletion. Subjects attended on three occasions and treatment phases were separated by a washout period of at least a week. Subjects were permitted a light breakfast prior to the commencement of each study, but were requested to refrain from all medications for 7 days, alcohol for 24 hours and caffeine for at least 4 hours prior to the start of the study. All subjects received 750 mg of the 11β-hydroxylase inhibitor, metyrapone, at midnight and 8 am on each occasion. Subjects attended the Wellcome Trust Clinical Research Facility for each study visit at 8 am, and rested recumbent throughout the study. A 17-G cannula was inserted into the antecubital vein of each arm as described previously (Plumpton *et al.* 1995), to allow blood sampling, and a dorsal foot vein was cannulated with a 23-G cannula for intravenous drug administration. At 8.30 am, subjects received either (i) intravenous saline placebo bolus and continuous infusion (absence of endogenous glucocorticoids), (ii) intravenous hydrocortisone 2.8 mg priming bolus and 1.8 mg/hr infusion (calculated to produce plasma cortisol ~300 nM, equivalent to physiological endogenous plasma glucocorticoid concentrations), or (iii) intravenous hydrocortisone 9.4 mg priming bolus and 6.1 mg/hr infusion (calculated to produce plasma cortisol concentrations ~1000 nM, ie supraphysiological glucocorticoid concentrations) in a randomised, double blind, placebo-controlled cross-over design. Two and a half hours after the commencement of the intravenous infusion of hydrocortisone or placebo, the brachial artery of the non-dominant arm was cannulated with a 27-G steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min.

Forearm studies were timed such that the basal forearm blood flow measurement was taken precisely 3 hours following the commencement of the intravenous hydrocortisone/placebo (i.e at approximately 11.30 am). Following a 30 minute equilibration period (during which intra-arterial saline was administered) subjects received intra-arterial infusions of bradykinin at 100, 300 and 1000 pmol/min (BK, an endothelium-dependent vasodilator which releases t-PA (Labinjoh *et al.* 2000; Labinjoh *et al.* 2001)), acetylcholine at 5, 10 and 20 μg/min (ACh, an endothelium-dependent vasodilator which does not release t-PA (Stein *et al.* 1998; Brown *et al.* 1999)) and sodium nitroprusside at 2, 4 and 8 μg/min (SNP, an endothelium-independent vasodilator which does not release t-PA (Jern *et al.* 1994b; Newby *et al.* 1997; Stein *et al.* 1998; Brown *et al.* 1999)) for 6-10 minutes at each dose. Forearm blood flow was measured three minutes after the start of each drug dose, and every 6-10 minutes throughout the washout periods. The three intra-arterial drug infusions were separated by 20 minute saline infusions and were administered in a random order, although this was kept constant for each individual subject. The study protocol is illustrated in Figure 5.1.

5.2.3 Drugs

Pharmaceutical grade bradykinin (BK; Merck Biosciences AG, Läufelfingen, Switzerland), acetylcholine (ACh, Novartis UK Ltd) and sodium nitroprusside (SNP, David Bull Laboratories, UK) were dissolved in physiological saline (0.9% sodium chloride: Baxter Healthcare Ltd, Thetford, UK) on the day of the study. Oral metyrapone (Metopirone®, Alliance Pharmaceuticals, UK) and intravenous hydrocortisone (dissolved in physiological saline; Solu-Cortef®, Pfizer UK) were administered according to the study protocol (Section 5.2.2, Figure 5.1).

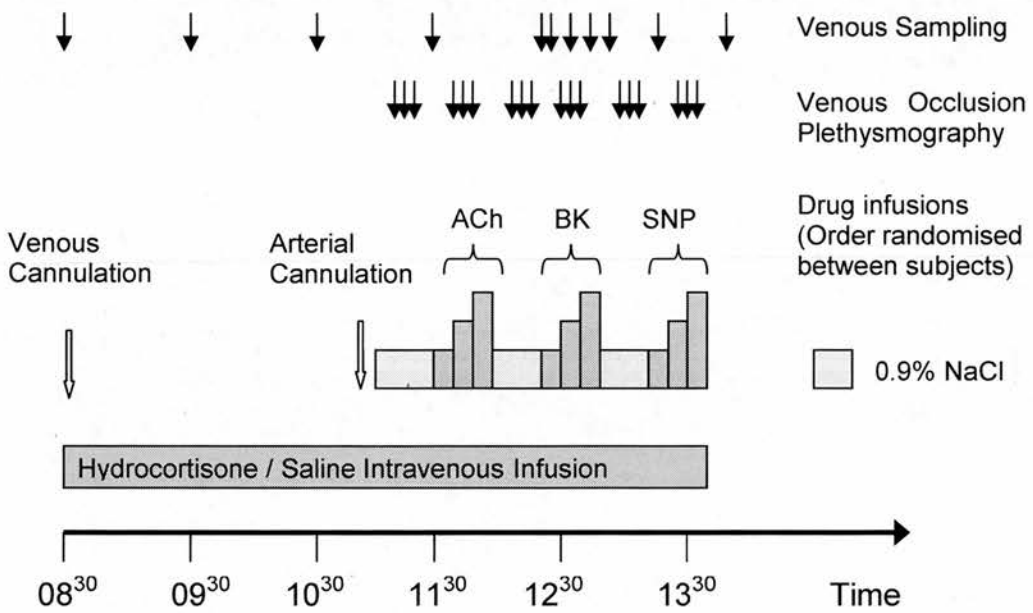

Figure 5.1 Study protocol

Subjects attended on three occasions and received intravenous placebo (0.9% saline), low dose hydrocortisone (2.8 mg bolus followed by 1.8 mg/hr) or high dose hydrocortisone (9.4 mg bolus followed by 6.1 mg/hr). The first intra-arterial drug infusion was commenced three hours after the start of the intravenous treatment. Venous sampling and venous occlusion plethysmography was performed as indicated. All subjects received 750 mg oral metyrapone at midnight and 8 am before each study. ACh = Acetylcholine, BK = bradykinin, SNP = sodium nitroprusside, NaCl = sodium chloride.

5.2.4 Haemodynamic measurements

Blood pressure and pulse were monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan)(Wiinberg *et al.* 1988).

The underlying principle of venous occlusion plethysmography is that if venous return from the forearm is occluded without impedance of arterial inflow, the forearm will swell in proportion to the rate of arterial inflow (Benjamin *et al.* 1995). This method measures forearm blood flow through skeletal muscle (50-70% of total) and skin. The hands must be excluded from the circulation as blood flow here is predominantly through arteriovenous shunts within the skin and this has different physiology from that of the forearm (Benjamin *et al.* 1995). Mercury-in-silastic strain gauges applied to the widest part of the forearm were used to measure changes in forearm circumference (Webb 1995). During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 200 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg pressure for 8 s in every 11 s to achieve venous occlusion and obtain plethysmographic recordings.

Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson) was processed by a PowerLab® analogue-to-digital converter and Chart™ *v*4.1.2 for Windows software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a portable computer (Figure 5.2). Calibration was achieved using the internal standard of the plethysmograph.

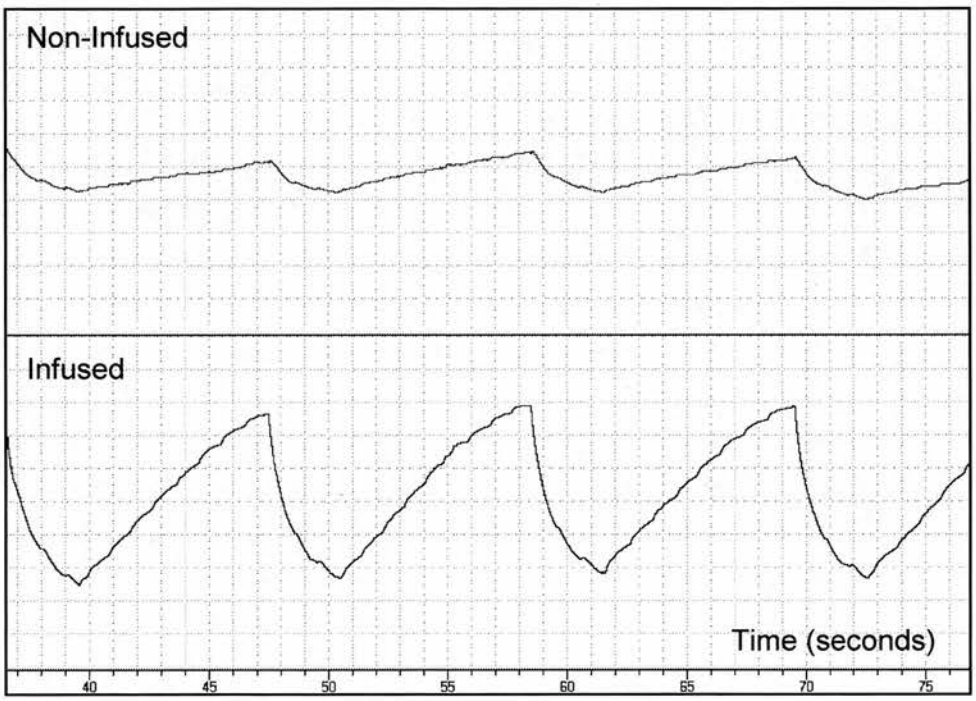

Figure 5.2 Representative plethysmography tracing

Representative trace (Chart™ *v*4.1.2 for Windows) from mercury-in-silastic strain gauges (converted to mV by analogue-to-digital converter) during intra-arterial bradykinin infusion. The slope from each cuff inflation period is used to calculate forearm blood inflow over the time period.

5.2.5 Venous sampling

Venous blood (8 ml) was withdrawn simultaneously from each arm at intervals throughout the study and collected into tubes containing potassium ethylene diamine tetraacetic acid (EDTA; Monovette®, Sarstedt, Nümbrecht, Germany), lithium heparin (Monovette®, Sarstedt, Nümbrecht, Germany), acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) and trisodium citrate (Monovette®, Sarstedt, Nümbrecht, Germany) for estimation of haematocrit, cortisol, t-PA, and PAI-1, respectively. Lithium heparin tubes were spun within 10 minutes at 2380 rpm for 10 minutes at 4°C. Citrate and stabilyte tubes were kept on ice before being centrifuged at 2,000 *g* for 30 minutes at 4°C. Platelet free plasma was decanted and stored at -80°C (Kluft *et al.* 1988).

5.2.6 Sample analysis

Biochemical screening assays were undertaken on venous samples obtained at screening visits by the hospital Clinical Laboratory facility (data not shown). Haematocrit was also determined by the hospital Clinical Laboratory facility using an automated Coulter counter (Beckman-Coulter ACt.8, High Wycombe, UK).

Plasma PAI-1 and t-PA antigen concentrations were determined using enzyme-linked immunosorbent assays (Elitest® for PAI-1 (Declerck & Collen 1990) and Zymutest® for t-PA (Booth *et al.* 1987), previously known as Coaliza®; Hyphen-Biomed, France). Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen respectively. The sensitivities of the assays were 1 ng/ml and 11 ng/ml, respectively.

Plasma cortisol concentrations were determined using an enzyme-linked immunosorbent assay (DRG Instruments GmbH, Germany) with <2% cross-reactivity for 11-deoxycortisol.

5.2.7 Data analysis and statistics

Plethysmographic data were extracted from the Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations. Forearm blood flow, expressed as millilitres per 100 ml forearm per minute, was calculated from the rate of increase in forearm circumference, using the internal calibration of the plethysmograph to convert the voltage changes resulting from changes in strain gauge length (mV) into circumference changes (mm) (Whitney 1953; Roddie & Wallace 1979). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs (Kerslake DMcK 1949; Webb 1995). Usually, the last five flow recordings in each three minute measurement period were calculated and averaged for each arm.

To reduce the variability of blood flow data, the ratio of flows in the two arms was also calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm (Benjamin *et al.* 1995; Webb 1995). Percentage changes in the infused forearm blood flow were calculated (Benjamin *et al.* 1995; Webb 1995) as follows:

$$\%\ Change\ in\ blood\ flow = 100 \times (I_t/NI_t - I_b/NI_b) / I_b/NI_b$$

where I_b and NI_b are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time point (t), respectively.

For the forearm studies, estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit, Hct and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused arms ($[t\text{-}PA]_{Non\text{-}inf}$):

$$Estimated\ net\ forearm\ t\text{-}PA\ release = FBF \times (1\text{-}Hct) \times ([t\text{-}PA]_{Inf} - [t\text{-}PA]_{Non\text{-}inf})$$

Values are expressed as mean ± standard error. Data were analysed using Student's t-tests, analysis of variance (ANOVA) or ANOVA with repeated measures followed by post-hoc tests, as appropriate. Statistical significance was taken at the 5% level.

5.3 Results

Oral, intravenous and intra-arterial drugs were well tolerated with no serious adverse events.

5.3.1 Plasma cortisol levels

An increase in plasma cortisol was evident following both low dose and high dose intravenous cortisol compared with placebo ($p<0.0001$; Figure 5.3).

5.3.2 Haemodynamic variables

Baseline (prior to intravenous drug administration) mean arterial pressure (MAP) and heart rate were similar on all three visits (Table 5.1). There was no change in pulse over the course of the study, although a significant rise in mean arterial pressure during the study days was noted in all three treatment groups (Figure 5.4; $p<0.001$).

Baseline forearm blood flows (at commencement of intra-arterial drug administration) were similar in infused and non-infused arms (Table 5.1). Although baseline blood flows did not differ significantly between placebo and hydrocortisone treatments, there was a trend towards a difference between groups ($p=0.08$ for non-infused arm).

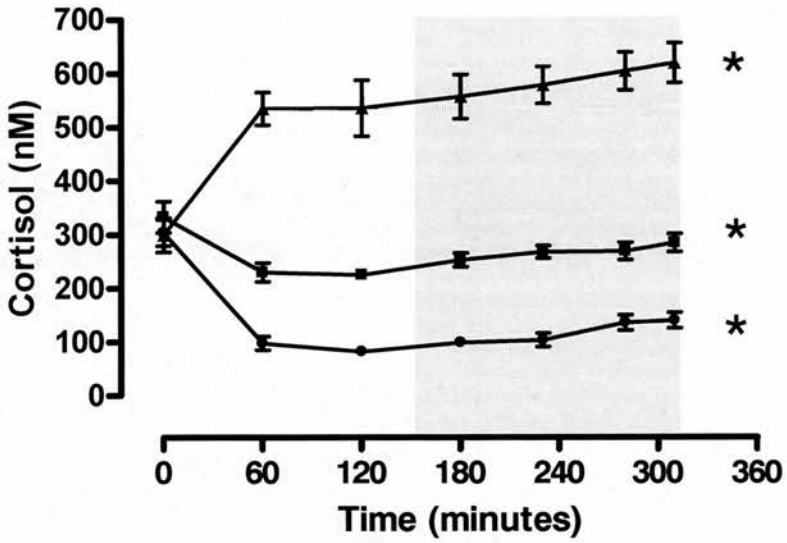

Figure 5.3 Changes in plasma cortisol

Plasma cortisol levels over the course of the study in subjects (n=12) treated with intravenous saline (closed circles), low dose hydrocortisone (closed squares) and high dose hydrocortisone (closed triangles). Time is recorded from commencement of intravenous infusion. Significant differences in plasma cortisol were observed between all three groups (*$p<0.0001$). Shaded area represents forearm plethysmography study.

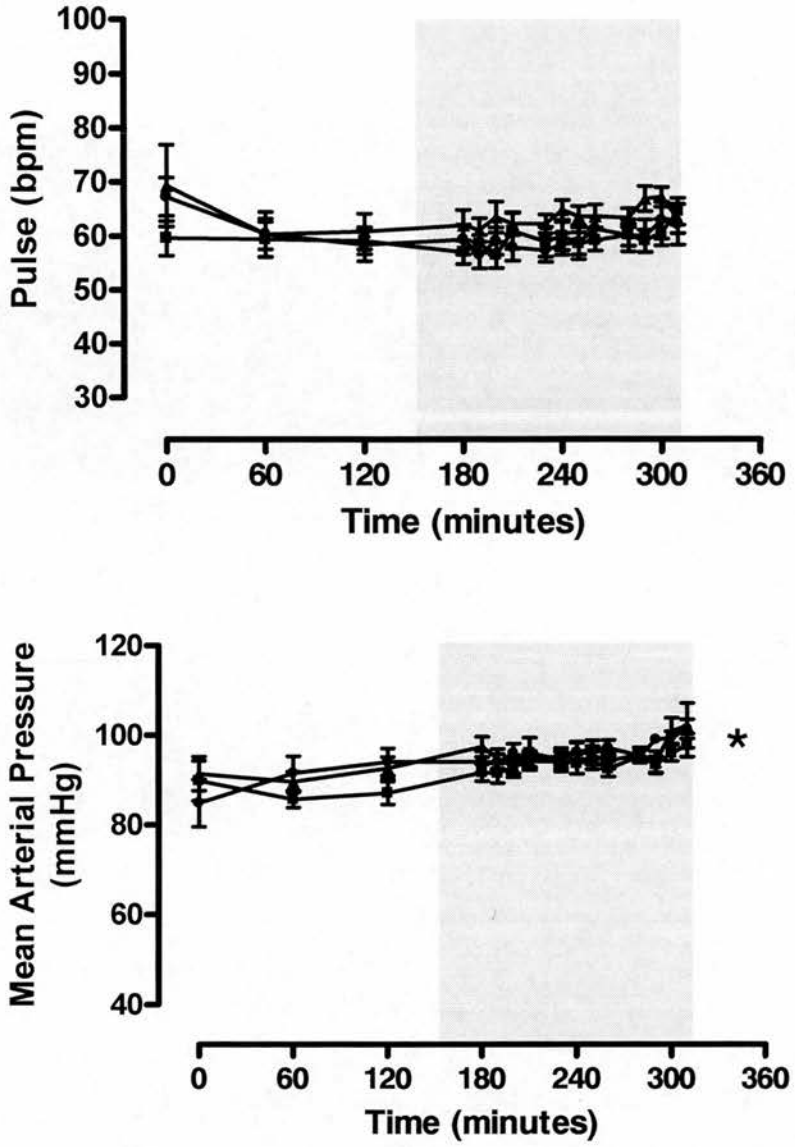

Figure 5.4 Haemodynamic variables

Pulse (beats per minute, bpm; A) and mean arterial pressure (MAP, mmHg; B) in subjects (n=12) treated with intravenous saline (closed circles), low dose hydrocortisone (closed squares) and high dose hydrocortisone (closed triangles). Time is recorded from commencement of intravenous infusion. There was no change in pulse during the study (p=0.10, by repeated measures ANOVA). A significant increase in MAP was observed in all three groups over the course of the study (*p<0.001, by repeated measures ANOVA). Shaded area represents forearm plethysmography study.

Table 5.1 Baseline haemodynamic characteristics

Measurement (units)	Placebo	Low dose Hydrocortisone	High dose Hydrocortisone	P value
Heart rate (/min)	67±3	63±3	68±4	0.30
Mean Arterial Pressure (mmHg)	88±4	89±3	92±2	0.59
Forearm blood flow, infused (ml/100ml/min)	2.0±0.3	3.0±0.5	2.4±0.3	0.12
Forearm blood flow, non-infused (ml/100ml/min)	1.8±0.2	2.8±0.4	2.3±0.3	0.08

Haematocrit values at baseline (prior to intravenous drug administration) were similar on all three visits (p=0.85, Table 5.2). There was a significant fall in haematocrit over the course of the study ($p<0.05$) although the absolute fall in haematocrit following intravenous hydrocortisone (reduction of 0.03±0.01 with either dose) did not differ significantly from placebo (reduction of 0.04±0.01, p=0.86).

Table 5.2 Haematocrit values

Haematocrit	**Placebo**	**Low dose Hydrocortisone**	**High dose Hydrocortisone**	**P value**
Baseline	0.45±0.01	0.45±0.01	0.44±0.01	0.85
End of study	0.41±0.01	0.42±0.01	0.41±0.004	0.38

5.3.3 Intra-arterial drug administration

Bradykinin (Figure 5.5), acetylcholine (Figure 5.6) and sodium nitroprusside (Figure 5.7) all caused dose-dependent increases in forearm blood flow. There were no significant changes in non-infused forearm blood flows.

Compared with placebo, administration of intravenous hydrocortisone caused no significant difference in the infused forearm blood flow during intra-arterial acetylcholine (Figure 5.6) or sodium nitroprusside (Figure 5.7). The percentage increase in forearm blood flow during intra-arterial bradykinin appeared blunted by hydrocortisone, at both low and high dose, although this did not reach statistical significance (p=0.08; Figure 5.5B).

5.3.4 Plasma fibrinolytic variables

Baseline plasma t-PA antigen concentrations were unchanged by systemic hydrocortisone infusion (Figure 5.8, Table 5.3). Bradykinin caused dose-dependent increases in infused arm t-PA concentrations which did not differ between treatment groups when comparing either actual t-PA release (p=0.74 for area under curve; Table 5.3) or estimated net t-PA release (p=0.88, Figure 5.8).

There was a trend towards an increase in infused arm baseline plasma PAI-1 concentrations with increasing intravenous hydrocortisone dose which did not reach statistical significance (p=0.10). However, in the non-infused arm, there was no difference between the groups (p=0.37; Table 5.3). There was no significant change in plasma PAI-1 concentrations throughout the study in either the infused (p=0.45) or non-infused (p=0.71) arm.

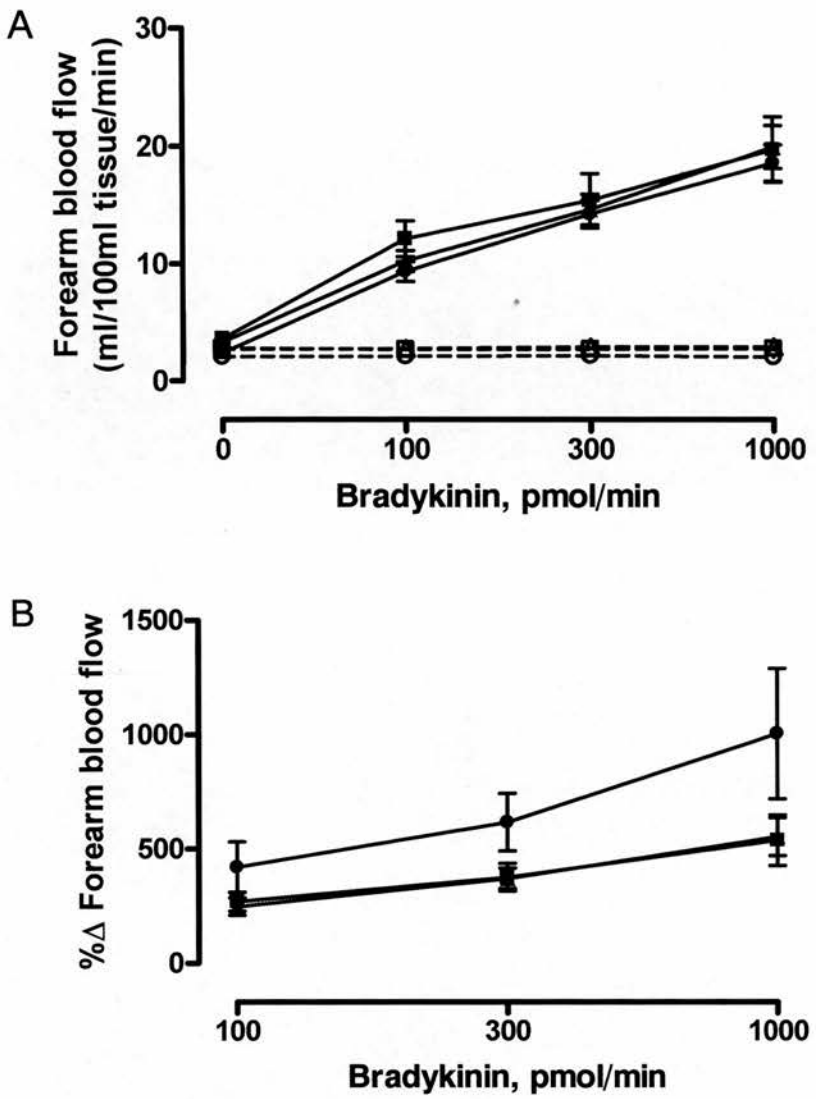

Figure 5.5 Forearm blood flow responses to bradykinin

Absolute (A) and percentage change (B) in infused (closed symbols) and non-infused (open symbols) forearm blood flows in response to increasing doses of bradykinin in subjects (n=12) treated with intravenous saline (circles), low dose hydrocortisone (squares) and high dose hydrocortisone (triangles). Intra-arterial bradykinin caused dose-dependent increases in forearm blood flow. There were no significant changes in non-infused forearm blood flows. The percentage increase in forearm blood flow during intra-arterial bradykinin appeared blunted by hydrocortisone, at both low and high dose, although this did not reach statistical significance (p=0.08).

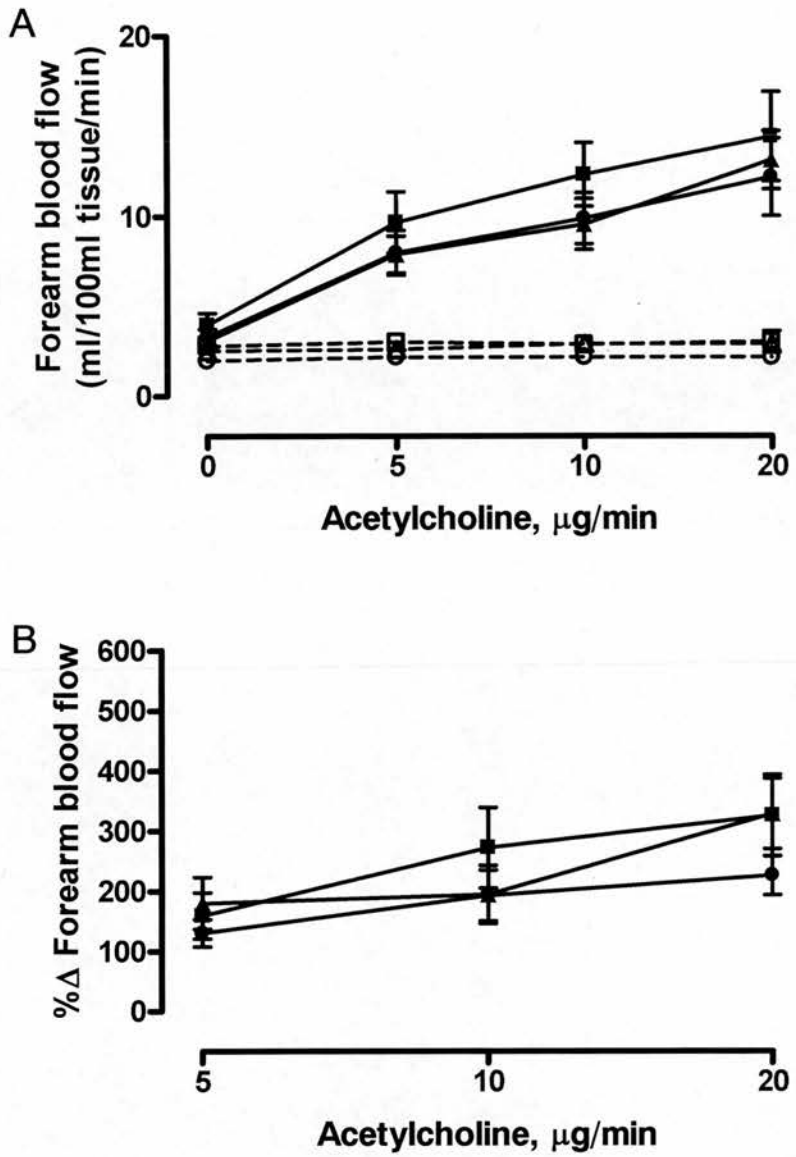

Figure 5.6 Forearm blood flow responses to acetylcholine

Absolute (A) and percentage change (B) in infused (closed symbols) and non-infused (open symbols) forearm blood flows in response to increasing doses of acetylcholine in subjects (n=12) treated with intravenous saline (circles), low dose hydrocortisone (squares) and high dose hydrocortisone (triangles). Intra-arterial acetylcholine caused dose-dependent increases in forearm blood flow. There were no significant changes in non-infused forearm blood flows. Compared with placebo, administration of intravenous hydrocortisone caused no significant difference in the infused forearm blood flow response to intra-arterial acetylcholine (p=0.18).

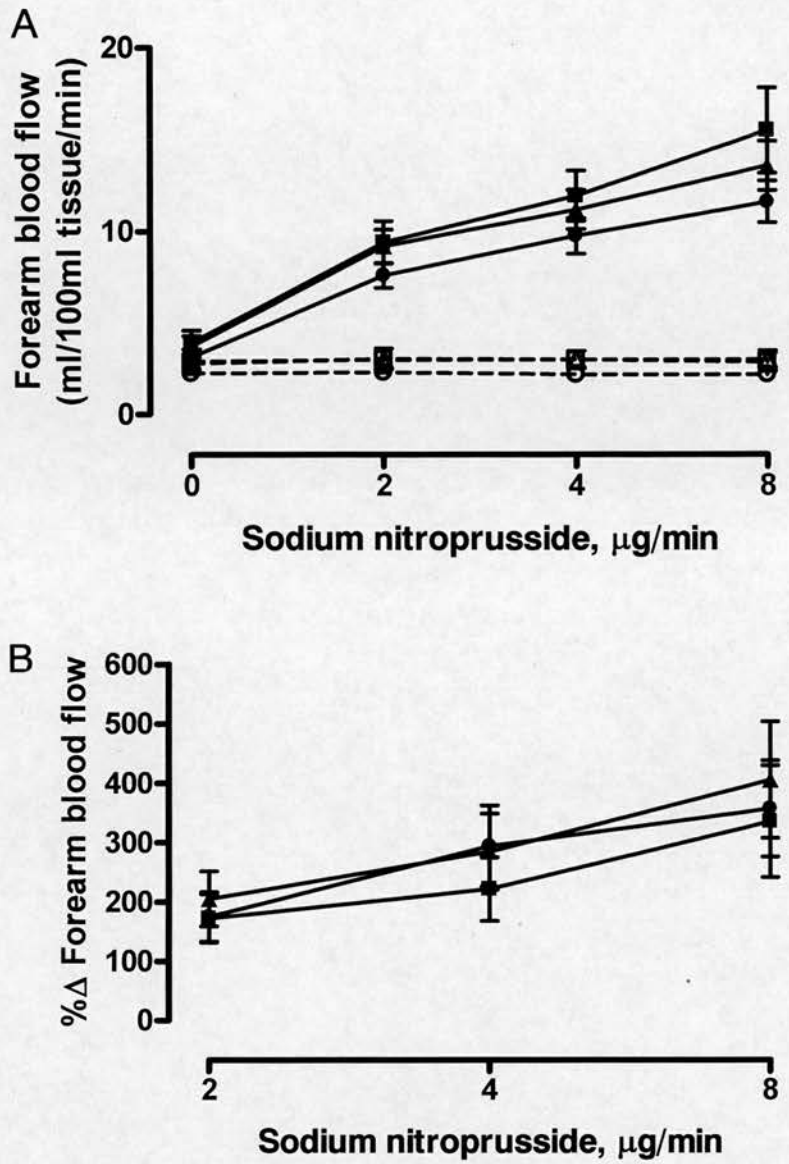

Figure 5.7 Forearm blood flow responses to sodium nitroprusside

Absolute (A) and percentage change (B) in infused (closed symbols) and non-infused (open symbols) forearm blood flows in response to increasing doses of sodium nitroprusside in subjects (n=12) treated with intravenous saline (circles), low dose hydrocortisone (squares) and high dose hydrocortisone (triangles). Intra-arterial sodium nitroprusside caused dose-dependent increases in forearm blood flow. There were no significant changes in non-infused forearm blood flows. Compared with placebo, administration of intravenous hydrocortisone caused no significant difference in the infused forearm blood flow response to intra-arterial sodium nitroprusside (p=0.19).

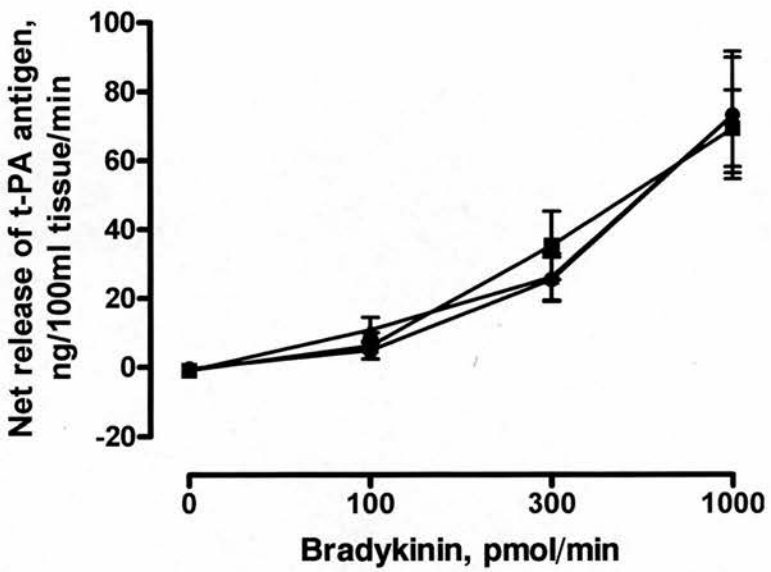

Figure 5.8 Estimated net t-PA release during intra-brachial bradykinin infusion

Net release of t-PA antigen during intra-brachial bradykinin infusion in subjects treated with intravenous saline (circles), low dose hydrocortisone (squares) and high dose hydrocortisone (triangles). Intra-arterial bradykinin caused dose-dependent increases in endothelial t-PA release which was not affected by intravenous hydrocortisone infusion when compared with saline placebo (n=12, p=0.88).

Table 5.3 Plasma t-PA and PAI-1 concentrations at baseline and during bradykinin administration, following intravenous placebo or hydrocortisone

Bradykinin, pmol/min	0	100	300	1000
Placebo				
Plasma t-PA antigen, ng/ml				
Infused arm	3.6±0.4	4.9±0.4	5.7±0.4	8.8±0.6
Non-infused arm	4.0±0.5	4.0±0.5	4.2±0.5	4.7±0.5
Plasma PAI-1 antigen, ng/ml				
Infused arm	18.8±1.7	-	-	18.5±1.6
Non-infused arm	19.3±1.0	-	-	19.9±2.7
Low dose hydrocortisone				
Plasma t-PA antigen, ng/ml				
Infused arm	3.7±0.3	4.7±0.4	5.9±0.3	8.4±0.4
Non-infused arm	4.0±0.3	4.1±0.3	4.3±0.3	4.8±0.3
Plasma PAI-1 antigen, ng/ml				
Infused arm	22.2±2.9	-	-	21.7±1.9
Non-infused arm	20.9±2.6	-	-	24.3±2.0
High dose hydrocortisone				
Plasma t-PA antigen, ng/ml				
Infused arm	3.7±0.3	4.7±0.4	5.3±0.4	7.8±0.6
Non-infused arm	3.7±0.4	4.0±0.4	3.6±0.3	4.4±0.4
Plasma PAI-1 antigen, ng/ml				
Infused arm	26.4±3.5	-	-	24.0±3.0
Non-infused arm	24.0±4.1	-	-	24.7±3.3

5.4 Discussion

The intention of these studies was to determine the effects of acute variations in glucocorticoid availability on endothelial cell function. The results demonstrate that, in contrast to prolonged administration of pharmacological doses of glucocorticoids (Mangos *et al.* 2000), short-term variations in circulating glucocorticoid levels, within the physiological range, do not alter endothelial cell vasomotor or fibrinolytic function *in vivo* in man.

Effects of cortisol on endothelium-dependent vasodilatation

Previous studies have shown that prolonged administration (5 days) of hydrocortisone results in hypertension and impairment of endothelium-dependent vasodilatation (Mangos *et al.* 2000). The effects of glucocorticoids on vascular responsiveness have been attributed to impairment of nitric oxide bioavailability (Mangos *et al.* 2000; Whitworth *et al.* 2002; Iuchi *et al.* 2003), although the exact mechanisms remain elusive. The findings that cortisol can induce hypertension within 24 hours (Whitworth *et al.* 2002; Williamson *et al.* 1996; Tam *et al.* 1997), and that ACTH infusion produces an elevation in blood pressure within 2-8 hours (Jackson *et al.* 2001) suggest that cortisol-mediated effects on vascular tone may occur through rapid non-genomic mechanisms. Thus it might be predicted that short-term variations in glucocorticoid availability would influence endothelial cell vasomotor function. However, the present study has demonstrated that this appears not to be the case, as no effect of variations in circulating cortisol levels on vasomotor function was evident. These results are consistent with a recently published study which found that systemic administration of hydrocortisone (at doses far in excess of those used in the present study: 200mg, infused over three hours) had no effect on plasma nitrate/nitrite activity or forearm vascular responsiveness to acetylcholine (Williamson *et al.* 2005). Additionally, a single dose of oral prednisolone (50mg) has been shown to have no effect on endothelium-dependent vasodilatation within 6 hours (Farquharson and Struthers 2002). Taken together with evidence that direct intra-arterial short-term cortisol infusion does not alter vascular

resistance in the human forearm (Williamson *et al.* 2005; van Uum *et al.* 2002a) or renal (van Uum *et al.* 2002b) vascular beds, these data collectively suggest that cortisol does not acutely alter basal vascular tone, nitric oxide generation or vasodilator responses.

Hydrocortisone infusion had no effect on either acetylcholine- or sodium nitroprusside-mediated vasodilatation. Absolute infused forearm blood flows during intra-arterial bradykinin (expressed as area under curve) were also similar in all three groups. However, upon "normalising" the forearm blood flow data, by calculating the percentage change in the ratios of the infused and non-infused flows, hydrocortisone infusion (at both low and high dose) appeared to impair bradykinin-mediated vasodilatation, although this did not reach statistical significance (p=0.08, Figure 5.5B). This result is difficult to interpret, however, as there were non-significant differences in basal forearm blood flow between the groups immediately prior to bradykinin administration (infused arm flows of 2.4±0.3, 3.6±0.6 and 3.3±0.5ml/100ml/min following placebo, low dose hydrocortisone and high dose hydrocortisone, respectively; p=0.06). When basal blood flows differ, the concentration of drug reaching the tissues vary and direct comparisons of vasodilator responses may not be valid (Benjamin *et al.* 1995; Pedrinelli *et al.* 1991). Furthermore, although the mean difference in percentage change in forearm blood flow during bradykinin infusion (1000 pmol/min) was 467% (for low dose hydrocortisone compared with placebo), the confidence intervals are wide and encompass zero (95% confidence intervals -148 to +1082%). For the study to have had an 80% power of detecting this magnitude of difference, it would have been necessary to increase the sample size to 42 subjects, which was not practicable. On balance therefore, considering the lack of effect of hydrocortisone on both acetylcholine- and bradykinin-mediated changes in absolute forearm blood flow, it is likely that acute variations in circulating glucocorticoids do not alter endothelium-dependent vasodilatation in the forearm.

As mentioned earlier, there was a trend towards an effect of hydrocortisone infusion on basal vascular tone with higher baseline forearm blood flows in the low dose

hydrocortisone group than either the placebo or high dose hydrocortisone group (Table 5.1). Whilst the magnitude of the difference in basal flows between low dose cortisol and placebo is of potential physiological relevance (1.0 ml/100 ml/min; 95% confidence intervals -2.4 to +3.4), the wide confidence intervals suggest significant variability in this response.

There are a number of possible explanations for the lack of effect of short term hydrocortisone infusion on endothelium-dependent vasodilatation. It may be that there are no direct effects of glucocorticoids on endothelial cell function in man. This conclusion is supported by previous studies which have shown that *in vitro* incubation of human subcutaneous resistance arteries with cortisol (30 μM for 3 hours) had no effect on endothelium-dependent vasoreactivity (Hadoke P *et al.*, unpublished observations). However, if glucocorticoid effects on nitric oxide bioavailability or other facets of vascular reactivity are mediated through *de novo* gene transcription, these may take many hours to become evident and might not have been detected by a study of this nature. For example, the excessive generation of reactive oxygen species and reduced nitric oxide availability which occur in human umbilical vein endothelial cells following incubation with dexamethasone are evident only after 6 hours (Iuchi *et al.* 2003). In contrast, however, other studies have demonstrated acute *in vivo* effects of glucocorticoids on the generation of reactive oxygen species by mononuclear cells (Dandona *et al.* 1998) and on muscle sympathetic nerve activity (Dodt *et al.* 2000) which are evident within 3 hours.

Another possible explanation for the lack of effect of variations in circulating glucocorticoids (within the physiological range) on vasomotor function is that 11βHSD activity within the vasculature may be sufficient to compensate for physiological variations in circulating concentrations of glucocorticoids achieved, resulting in "damping" of the vascular response to the fluctuations in circulating glucocorticoid levels achieved in this study. This tissue-specific regulation of glucocorticoid action is the central premise on which this thesis is based. However, this hypothesis is not supported by previous work which has shown that *in vivo* administration of the non-selective 11βHSD inhibitor carbenoxolone has no effect on

endothelium-dependent vasoreactivity in man (Hadoke P *et al.*, unpublished observations). Furthermore, the data presented in Chapters 3 & 4 indicate the predominant regeneration of glucocorticoids by the 11βHSDs within murine vascular tissue. If 11β-reduction is also the predominant reaction direction within human vasculature, then the 11βHSDs would be expected to amplify, rather than dampen, intracellular glucocorticoid action in the vasculature. This debate highlights the challenges that are faced when undertaking translational research. Studies using transgenic 11βHSD2-/- mice have allowed investigation of glucocorticoid effects *in vitro* in a situation when absolute vascular glucocorticoid levels can be predicted as there is no inactivation of glucocorticoids (Christy *et al.* 2003). However, the present study was conducted in an attempt to extend these studies to examine these glucocorticoid effects in humans *in vivo*. There are as yet no selective inhibitors of the isozymes of 11βHSD available for use in man and, therefore, it is not possible to examine the influences of glucocorticoids in man *in vivo* without the need to consider tissue-specific variations in glucocorticoid availability as a result of 11βHSD activity. Nevertheless, if the negative findings here are explained by "damping" of physiological variations in glucocorticoids by 11βHSD activity, then this is an important *in vivo* component of the question as to whether endogenous variations in glucocorticoids influence endothelial cell function.

It is worth considering that metyrapone was administered to all subjects in each treatment phase. Whilst it is possible that this drug had an effect on endothelial function which confounded the effects of glucocorticoids, for example due to altered levels of the precursor mineralocorticoid 11-deoxycortisol between groups (due to differing feedback inhibition of ACTH by cortisol in the three groups), this is unlikely as previous studies have shown no effect of this drug on either basal forearm blood flow in vivo or endothelial nitric oxide release in vitro (Broadley *et al.* 2005) .

Effects of hydrocortisone on endothelial cell fibrinolytic function

Although glucocorticoid excess has been associated with elevated circulating PAI-1 concentrations and also with a hypercoagulable state (Sartori *et al.* 2000; Fatti *et al.*

2000; Patrassi *et al.* 1985; Ikkala *et al.* 1985; Patrassi *et al.* 1992; Sartori *et al.* 1999), there have been no previous studies which have investigated whether physiological variations in glucocorticoid concentrations influence the local fibrinolytic capacity of the endothelium.

The present study has demonstrated that short term systemic administration of hydrocortisone has no effect on acute fibrinolytic capacity. Variations in circulating glucocorticoid levels had no effect on plasma PAI-1 concentrations and no effect on basal or bradykinin-stimulated release of t-PA from the endothelium. Taken together with the lack of clear effect of acute hydrocortisone administration on endothelium-dependent vasodilatation, these results support the conclusion that short term changes in plasma glucocorticoid concentrations, within the physiological range, do not impair endothelial cell function. The mechanisms regulating acute t-PA release and plasma PAI-1 concentrations remain to be established but are likely to involve the nitric oxide pathway (Newby *et al.* 1998). Augmentation of t-PA release is seen following local and systemic inflammation (Chia *et al.* 2003b; Chia *et al.* 2003a), and impairment of t-PA release, evident in smokers (Newby *et al.* 2001), is predictive of increased cardiovascular risk (Meade *et al.* 1993). Whilst the present study has shown that there is no effect of short term glucocorticoid administration on bradykinin-stimulated endothelial cell t-PA release, acute t-PA release can also be stimulated by other agonists such as substance P and methacholine through different mechanisms, thus it remains possible that glucocorticoids influence alternative pathways of t-PA release. However, the vaosomotor and fibrinolytic results are concordant, with a lack of effect of hydrocortisone on both vasodilatation and acute t-PA release, making it likely that this negative finding is robust.

In addition to the lack of effect of hydrocortisone on acute t-PA release, the present study also demonstrated that there was no significant change in plasma PAI-1 concentrations following short term hydrocortisone administration. These results parallel those from a recent study which showed no change in plasma PAI-1 concentrations in healthy volunteers treated with dexamethasone (6 mg) daily for 5 days (Brotman *et al.* 2005). However, elevations in plasma PAI-1 concentrations

have been previously demonstrated in patients with endogenous glucocorticoid excess (Fatti *et al.* 2000) and in those receiving long term glucocorticoid therapy following heart (Sartori *et al.* 1999) and renal transplantation (Patrassi *et al.* 1985; Patrassi *et al.* 1992). These elevations in PAI-1 improve upon surgical cure (Fatti *et al.* 2000) or steroid withdrawal (Sartori *et al.* 2000). Furthermore, studies *in vitro* (Gelehrter *et al.* 1987; Udden *et al.* 2002; Fukumoto *et al.* 1992; Reinders *et al.* 1992; Halleux *et al.* 1999; Morange *et al.* 1999; Uno *et al.* 1998), and in animal models *in vivo* (van Giezen & Jansen 1992; van Giezen *et al.* 1994) have also demonstrated an augmentation of PAI-1 release following glucocorticoid treatment. The lack of effect of short term hydrocortisone on plasma PAI-1 concentrations in this study suggests that, in contrast to studies of chronic steroid excess, or studies *in vitro*, PAI-1 is not released in response to hydrocortisone over the time course and at the physiological doses used in the present study.

Conclusions

In summary, these data suggest that short-term variations in plasma glucocorticoid concentrations within the physiological range do not alter endothelial cell fibrinolytic or vasomotor function. It may be that there are either no direct effects of glucocorticoids on endothelial cell function *in vivo*, or that they require a longer duration to become manifest. Alternatively, within the range of glucocorticoid concentrations studied here, the vascular 11βHSDs may provide a "damping" mechanism which regulates exposure of the endothelium to glucocorticoids. However, the negative results from this study should not be over-interpreted. A key hypothesis of this thesis was that inflammation alters 11βHSD activity to favour increased glucocorticoid availability. It remains a possibility that whilst up-regulation of 11β-reductase activity by inflammatory mediators may not be relevant under physiological conditions, it may become important during pathophysiological disease processes. The present study aimed to determine whether changes in glucocorticoid availability (that might result from inflammation-mediated changes in 11βHSDs) would impair endothelial cell function in man *in vivo*. However, the technique of systemic manipulation of glucocorticoids to address this is limited in

part by the fact that intracellular glucocorticoid levels may be modulated by the activity of the 11βHSDs. Therefore, it remains a possibility that pathological insults which alter vascular 11βHSD activity may have consequences for endothelial cell function. The emergence of pharmaceutical preparations of selective inhibitors of the isozymes of 11βHSD will allow more detailed exploration of the influences of glucocorticoids on vascular function *in vivo*.

Chapter Six

Conclusions and Future Directions

It is known that glucocorticoids directly influence many aspects of vascular function, and that these effects are mediated, at least in part, by the isozymes of 11βHSD. Inflammation, a key component of many vascular disease processes, up-regulates 11βHSD1 in cultured human smooth muscle cells, but whether this process occurs in intact arteries had not been explored previously. The research presented in this thesis aimed to determine whether inflammatory mediators influence local metabolism of glucocorticoids in intact vascular tissue, and whether the resultant changes in glucocorticoid availability impair endothelial cell function.

The activity, expression and localisation of the 11βHSD isozymes in vascular tissue have been extensively investigated. However, the relative contributions of dehydrogenase and reductase activities, and the directionality of each isozyme within intact vascular tissue had not been clearly elucidated. The first hypothesis contained in this thesis was that both 11βHSD1 and 11βHSD2 activities are present in the intact vessel, where they act, as exclusive reductase and dehydrogenase, respectively, to regulate local availability of glucocorticoids. To address this question, novel *in vitro* and *in vivo* assays were developed to measure 11β-reductase and dehydrogenase activities independently. In Chapter 3, an *in vitro* intact artery assay was used to demonstrate that both 11β-reductase and dehydrogenase activities are present in both aorta and iliofemoral arteries from wild type mice, and that the reductase direction predominates by approximately 10-fold. Moreover, regional differences in 11β-reductase activity were noted, with higher activity in the aorta than the iliofemoral vessels. Use of mice with genetic inactivation of either 11βHSD1 or 11βHSD2 made it possible to determine the reaction direction for each isozyme. 11βHSD2 was shown to act as an exclusive dehydrogenase whilst 11βHSD1 appeared to have bi-directional activity *in vitro*. As there has been much debate as to whether 11βHSD1 acts as both reductase and dehydrogenase *in vivo*, it was necessary clarify this contentious issue by extending the studies of isozyme directionality into an *in vivo* model. Studies presented in Chapter 4 used a hindquarter perfusion model to assess 11βHSD activity *in vivo* in a regional perfused bed. As with the *in vitro* studies, 11β-reduction was the predominant reaction direction. However, in contrast to the *in vitro* studies, no 11β-dehydrogenase activity was evident in the perfused hindquarter of

11βHSD2-/- mice. Thus it is likely that 11βHSD1 acts as a predominant reductase in intact vascular tissue. The dehydrogenase activity attributed to 11βHSD1 *in vitro* is likely to be due to liberation of this isozyme from cells damaged during preparation and sectioning of the artery.

Inflammatory cytokines up-regulate 11βHSD1 activity and expression in cultured human aortic smooth muscle cells, but their influence on 11βHSDs in intact vessels had not been previously evaluated. Studies described in Chapter 3 addressed the question of whether inflammatory mediators up-regulate 11βHSD1 activity in intact arteries *in vitro*. Although it was possible to demonstrate that cytokines up-regulate 11βHSD1 in cultured murine aortic smooth muscle cells, there was no such effect of individual cytokines on 11βHSD1 or 11βHSD2 activity in intact aortic rings *in vitro*. However, a modest increase in 11β-reductase activity in aortic rings from mice who had received *in vivo* LPS suggested that there may be factors present *in vivo,* but not *in vitro,* which are required for the inflammatory regulation of 11βHSD1 to become manifest. To explore this further, under more physiological conditions, studies in Chapter 4 were performed using the hindquarter perfusion model. Consistent with the data from Chapter 3, there was no effect of LPS on 11βHSD1 activity in the perfused hindquarter suggesting that up-regulation of 11β-reductase activity is unlikely to be a significant accompaniment of vascular inflammation in healthy arteries *in vivo*. However, it remains possible that 11βHSD1 may be up-regulated in pathological conditions associated with intense cell proliferation, such as vessel injury or atheroma, or in the context of augmented glucocorticoid availability (e.g., stress, acute illness). Thus, although up-regulation of 11β-reductase activity by inflammatory mediators may not be relevant under physiological conditions it may become important during pathophysiological disease processes.

Regulation of glucocorticoid activity within the arterial wall has the potential to influence many aspects of vascular structure and function. Elevations in circulating glucocorticoid concentrations, and even variations in the normal range in healthy individuals, are associated with increased cardiovascular risk. This association may be linked to the ability of glucocorticoids to directly impair endothelial cell function.

However, the effects of acute changes in glucocorticoid availability on endothelium-dependent vasodilatation and fibrinolytic function in man have not been explored previously. The final hypothesis in this thesis was that acute variations in glucocorticoid availability would mediate impaired endothelial function, an early marker of vascular disease. The study described in Chapter 5 used the forearm venous occlusion plethysmography technique to investigate whether short-term changes in circulating glucocorticoid levels altered endothelium-dependent vasodilatation or the fibrinolytic capacity of the endothelium. The results from this study suggest that, in contrast to previously demonstrated effects of prolonged glucocorticoid administration, acute changes in systemic glucocorticoid levels do not alter endothelial cell function. This suggests that the effects of glucocorticoids on endothelial cell function either take longer than 3 hours to become manifest, or are indirect. A speculative explanation for this negative finding may lie in the exquisite 11βHSD system, central to this thesis, whereby, despite fluctuations in circulating glucocorticoid concentrations, vascular intracellular glucocorticoids levels are tightly regulated.

To summarise, the studies presented in this thesis demonstrate that both 11βHSD1 and 11βHSD2 activities are present in intact arteries where they act, as reductase and dehydrogenase respectively, to modulate local glucocorticoid concentrations. Although inflammatory up-regulation of 11βHSD1 is not evident in healthy intact vascular tissue, it should not be inferred that the enzyme does not play a role in modulating glucocorticoid signalling since the current studies demonstrate the predominant regeneration of glucocorticoids within the vessel wall. Moreover, in pathological conditions associated with intense cell proliferation, such as vessel injury or atheroma, it remains possible that 11βHSD1 is up-regulated. Acute systemic variations in glucocorticoid availability do not impair endothelial cell function, although it remains to be seen whether intracellular changes in glucocorticoids cause endothelial cell dysfunction.

These studies have provided a basis for future studies to establish the influence of glucocorticoid regeneration on the proliferative and inflammatory responses within

the blood vessel. Unexpected differences between the effects of cytokines on 11βHSD1 activity in cultured cells and in intact vessels highlighted by the present studies deserve further exploration, and, to exclude the possibility that there are species differences in the inflammatory regulation of 11βHSD1, future work should include attempts to recapitulate published data showing cytokine-mediated upregulation of 11βHSD1 in human smooth muscle cells. Also, it will be important to examine the significance of alterations in smooth muscle cell proliferation or differentiation, and endogenous glucocorticoid tone, on the responsiveness of 11βHSD1 to inflammatory mediators. Studies using models of accelerated atherosclerosis and femoral artery injury are already underway in our laboratories to ascertain the effects of inflammatory mediators on vascular 11βHSD activity under pathological conditions. Also, the possibility that inflammation alters glucocorticoid metabolism and paracrine signalling from perivascular adipose tissue will be examined. Finally, with the development of selective inhibitors of human 11βHSDs, there is the opportunity to examine the effects of local manipulation of glucocorticoid metabolism on endothelial cell function.

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Appendix One

Publications

Date: Thu, 15 Sep 2005 14:10:09 -0400 [15/09/05 07:10:09 PM GMT]

From: "Millerd, Tiffany" <TMillerd@nas.edu>

To: Anna R Dover <Anna.Dover@ed.ac.uk>

Subject: RE: Request for Permission to Reprint

Dear Ms. Dover,

Re: Gary R. Small, Patrick W. F. Hadoke, Isam Sharif, Anna R. Dover, Danielle Armour, Christopher J. Kenyon, Gillian A. Gray, and Brian R. Walker Preventing local regeneration of glucocorticoids by 11-hydroxysteroid dehydrogenase type 1 enhances angiogenesis PNAS 2005 102: 12165-12170

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Best regards,
Tiffany Millerd for
Diane Sullenberger
Executive Editor
PNAS

Preventing local regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 enhances angiogenesis

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Edited by Judah Folkman, Harvard Medical School, Boston, MA, and approved June 30, 2005 (received for review January 25, 2005)

Angiogenesis restores blood flow to healing tissues, a process that is inhibited by high doses of glucocorticoids. However, the role of endogenous glucocorticoids and the potential for antiglucocorticoid therapy to enhance angiogenesis is unknown. Using *in vitro* and *in vivo* models of angiogenesis in mice, we examined effects of (*i*) endogenous glucocorticoids, (*ii*) blocking endogenous glucocorticoid action with the glucocorticoid receptor antagonist RU38486, and (*iii*) abolishing local regeneration of glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1). Glucocorticoids, administered at physiological concentrations, inhibited angiogenesis in an *in vitro* aortic ring model and *in vivo* in polyurethane sponges implanted s.c. RU38486-enhanced angiogenesis in s.c. sponges, in healing surgical wounds, and in the myocardium of mice 7 days after myocardial infarction induced by coronary artery ligation. 11βHSD1 knockout mice showed enhanced angiogenesis *in vitro* and *in vivo* within sponges, wounds, and infarcted myocardium. Endogenous glucocorticoids, including those generated locally by 11βHSD1, exert tonic inhibition of angiogenesis. Inhibition of 11βHSD1 in liver and adipose has been advocated to reduce cardiovascular risk in the metabolic syndrome: these data suggest that 11βHSD1 inhibition offers a previously uncharacterized therapeutic approach to improve healing of ischemic or injured tissue.

myocardial infarction | wound healing

Angiogenesis, the formation of new vessels from existing ones, is a key factor in many common diseases (1–4), and manipulation of angiogenesis is an important therapeutic target (5, 6). Supraphysiological concentrations of glucocorticoids have been used *in vitro* and *in vivo* to inhibit angiogenesis (7–11). It is unknown, however, whether physiological concentrations of endogenous glucocorticoids (principally cortisol in humans and corticosterone in mice) regulate angiogenesis.

The influence of glucocorticoids on their target tissues is regulated in a tissue-specific manner by the isozymes of 11β-hydroxysteroid dehydrogenase (11βHSD) (12). 11βHSD type 1 functions predominantly as an 11-oxidoreductase converting inactive 11-keto metabolites (cortisone in humans; 11-dehydrocorticosterone in mice) into active 11-hydroxy glucocorticoids (cortisol and corticosterone) (13). 11βHSD-1 is highly expressed in liver, adipose tissue, and regions of the central nervous system, where it amplifies intracellular glucocorticoid concentrations and thereby maintains glucocorticoid receptor activation (13). 11βHSD type 2 is an exclusive 11β-dehydrogenase, inactivating cortisol or corticosterone in distal nephron, colon, and sweat glands, thus preventing inappropriate access of glucocorticoids to mineralocorticoid receptors (12). Both 11βHSD isozymes are expressed in the blood vessel wall (14–18). In mouse and rat aorta, 11βHSD-2 is localized in endothelial cells and 11βHSD-1 in vascular smooth muscle (15, 16).

Glucocorticoids have diverse effects on vascular function, altering vasoconstrictor responses (19), impairing endothelium-dependent vasodilatation (19), and inhibiting inflammation and cell proliferation (20, 21). We recently reported studies of vascular function in knockout mice deficient in either 11βHSD isozyme (22). In aortae from 11βHSD-2 −/− mice, endothelium-dependent vasodilatation was impaired, suggesting that 11βHSD-2 protects endothelial cell receptors from glucocorticoids. However, there was no abnormality of vascular tone in 11βHSD-1 −/− mice, so the role of the type 1 isozyme in the vessel wall remained unclear. At that time, Cai *et al.* (23) demonstrated that 11βHSD1 expression in vascular smooth muscle is up-regulated in response to proinflammatory cytokines, raising the possibility that increased local generation of glucocorticoids contributes to feedback regulation of vascular inflammation.

Given that inflammatory cytokines can promote angiogenesis (24) and pharmacological doses of glucocorticoids have antiangiogenic activity, we hypothesized that 11βHSD-1 modulates angiogenesis by determining the local regeneration of active glucocorticoid in the vessel wall. If so, then manipulation of 11βHSD-1 may provide a novel therapeutic tool to alter angiogenesis. Here, we have tested this hypothesis by using *in vitro*, *in vivo*, and pathological models of angiogenesis in mice.

Methods

Mice. Male, C57Bl6J wild-type and 11βHSD-1 homozygous null (−/−) mice aged 8–10 weeks were used (Charles River Laboratories). Genetic inactivation of 11βHSD-1 has been described in MF-1/129 mice (25); for the current experiments, mice were backcrossed over >10 generations onto a C57Bl6J background (26).

Aortic Ring Preparations. Mice were killed, and thoracic aortae were removed, washed in serum-free MCDB 131 medium (Invitrogen), cleaned of periadventitial tissue, and divided into 1- to 3-mm rings.

11βHSD activities were measured by incubating wild-type aortic rings for 24 h at 37°C in 1 ml of DMEM-F12 medium (Invitrogen) containing ^{3}H-steroid supplemented with FBS (1%), streptomycin (100 μg/ml), penicillin (100 units/ml), and amphotericin (0.25 μg/ml) (27). 11β-Reductase activity was determined by adding 10 pmol [$^3H_4$]-11-dehydrocorticosterone [synthesized in-house from 1,2,6,7-[$^3H_4$]-corticosterone (Amersham Pharmacia Biosciences) by using rat placental homogenate]. Mouse liver (28 ± 5 mg) and medium alone were used as positive and negative controls, respectively. 11β-Dehydrogenase activity was determined by adding 10 pmol 1,2,6,7-[$^3H_4$]-corticosterone. Mouse kidney (13 ± 3 mg) and medium alone served as positive and negative controls. After incubation,

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Abbreviation: 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1.

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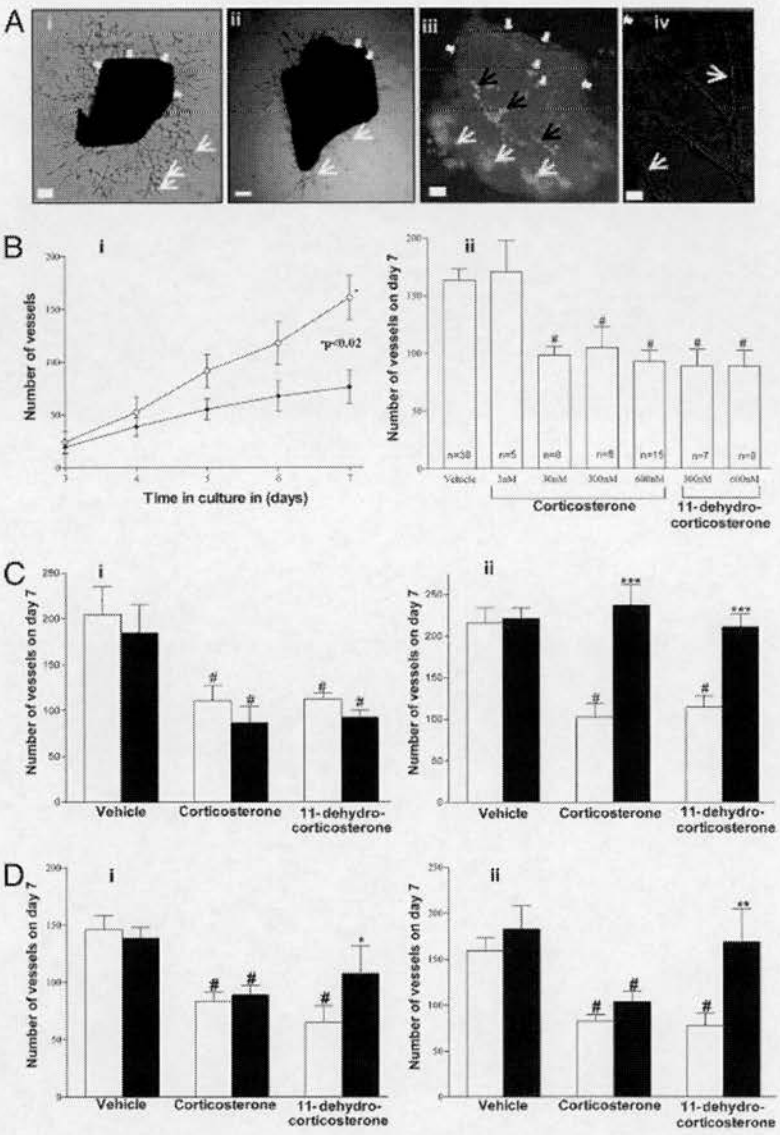

Fig. 1. Angiogenesis in aortic rings *in vitro*. (*A*) Light microscopy of new vessels shown sprouting from aortic rings. (*i*) Aortic ring incubated for 7 days without glucocorticoid. (*ii*) Aortic ring incubated for 7 days in the presence of glucocorticoid. Thick white arrows indicate the aortic ring; thin white arrows indicate new vessels. (Scale bar: 0.2 mm.) (*iii*) Uptake of low-density lipoprotein (LDL) is shown by fluorescence microscopy. This ring was incubated for 7 days without steroids. Thick white arrows indicate the aortic ring; thin white arrows indicate uptake of fluorescent labeled LDL in endothelial cells in new vessels; black arrows indicate uptake in endothelial cells of the aortic ring. (Scale bar: 0.2 mm.) (*iv*) High power view of new vessels; thick white arrows indicate the aortic ring and thin white arrows indicate uptake of fluorescent labeled low density lipoprotein in endothelial cells (Scale bar: 0.02 mm.) (*Bi*) Time course and effect of corticosterone on angiogenesis. ○, results from vessels incubated without steroids; ◆, results from vessels incubated with corticosterone (600 nM). Results are mean ± SEM for $n = 4$ per group. Comparison was by repeated measures ANOVA; *, $P < 0.02$. (*Bii*) Effects of corticosterone and 11-dehydrocorticosterone. Vessels were counted after 7-day incubation with steroids. Results are mean ± SEM. #, $P < 0.01$ versus vehicle by 2-way ANOVA and least squares difference post hoc test. (*C*) Influence of receptor antagonism. (*i*) Effects of the mineralocorticoid receptor antagonist spironolactone. Aortic rings from C57Bl6J mice were incubated with (filled bars) and without (open bars) spironolactone (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for $n = 6$ experiments. #, $P < 0.02$ versus corresponding vehicle. Spironolactone alone had no effect. (*ii*) Effects of the glucocorticoid receptor antagonist RU38486. Aortic rings from C57Bl6 mice were incubated with (filled bars) and without (open bars) RU38486 (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for $n = 4$–6 experiments. # $P < 0.01$ versus corresponding vehicle. ***, $P < 0.001$ for the effect of RU38486 in the presence of glucocorticoid. RU38486 alone had no effect. (*D*) Effects of 11βHSD inhibition (*i*) Pharmacological inhibitor carbenoxolone. Aortic rings from C57Bl6J mice were incubated with (filled bars) and without (open bars) carbenoxolone (10^{-6} M) and glucocorticoids (600 nM). Results are mean ± SEM for $n = 5$ experiments. #, $P < 0.01$ versus corresponding vehicle. *, $P < 0.04$ for the effect of carbenoxolone in the presence of 11-dehydrocorticosterone. Carbenoxolone had no effect in the presence of corticosterone or vehicle alone. (*ii*) Transgenic deletion of 11βHSD1. Effects of corticosterone and 11-dehydrocorticosterone on angiogenesis in vessels from 11βHSD1 −/− mice. Aortic rings from C57Bl6J wild-type (open bars) or 11βHSD1 −/− (filled bars) mice were incubated with and without glucocorticoids (600 nM). Results are mean ± SEM for $n = 7$ experiments. #, $P < 0.01$ versus corresponding vehicle. **, $P < 0.01$ for differences in angiogenesis between wild-type and 11βHSD1 −/− mice. Angiogenesis was not different between strains in the presence of vehicle or corticosterone but was inhibited by 11-dehydrocorticosterone in wild-type but not 11βHSD1 −/− mice.

steroids were extracted from media by using Sep-Pak C_{18} columns (Waters Millipore). Aortic rings, which contain only 2–3% of the added radioactivity, were not included in the extraction (27). [$^3H_4$]-Corticosterone and [$^3H_4$]-11-dehydrocorticosterone were separated by HPLC and quantified by on-line liquid scintillation counting (16). Enzyme activity was expressed as conversion after subtraction of apparent conversion in negative control wells. Both 11β-reductase (0.65 ± 0.24 pmol/mg) and 11β-dehydrogenase (0.66 ± 0.28 pmol/mg) activities were detected in aortic rings with similar conversion rates as in positive controls: liver for 11βHSD-1 (0.18 ± 0.03 pmol/mg) and kidney for 11βHSD-2 (2.13 ± 1.65 pmol/mg).

To quantify angiogenesis, aortic rings were embedded in 200 μl of steroid-free Matrigel (Becton Dickinson) (Fig. 1) and incubated at 37°C in serum-free MCDB 131, with heparin, ascorbic acid, and GA1000 (Cambrex Biosciences) in the presence and absence of corticosterone (3, 30, 300, and 600 nM), 11-dehydrocorticosterone (300 and 600 nM), the glucocorticoid receptor antagonist RU38486 (10^{-6} M), the mineralocorticoid receptor antagonist spironolactone (10^{-6} M), and/or the nonselective 11βHSD inhibitor carbenoxolone (10^{-6} M). All drugs (Sigma-Aldrich) were dissolved in ethanol and diluted in aqueous solution; final ethanol concentration 1–3% vol/vol. Media were changed every 48 h. Experiments were performed in triplicate. In initial experiments, new vessels were counted daily by using light microscopy (ref. 8 and Fig. 1). From these studies, day 7 was selected as the appropriate time point to examine the effects of glucocorticoids (Fig. 1*B*).

To confirm the nature of apparent new vessels, endothelial cells were identified by uptake of fluorescent-labeled acetylated low-density lipoprotein (Biogenesis, Poole, U.K.) (Fig. 1*A*).

s.c. Sponge Implant Assay. Mice were anesthetized with halothane, and a sterilized sponge cylinder (0.5 cm × 1 cm) (Caligen Foam, Accrington, Lancashire, U.K.) was implanted s.c. on each flank. Sponges contained a silastic insert (Silastic 20 medical grade, Dow Corning) impregnated with vehicle, 2 mg of cortisol or

cortisone, or 5.25 mg of RU38486. Each animal had an intervention-impregnated sponge (steroid or RU38486) on one side and a placebo-impregnated sponge (silastic only) on the other. Such inserts release their impregnated compounds *in vivo* at a constant rate for 3 weeks (28). Human steroids (cortisol and cortisone, equivalent to corticosterone and 11-dehydrocorticosterone) were used to allow distinction from endogenous steroids. In separate experiments (data not shown), angiogenesis in placebo-impregnated sponges was not altered by the presence or absence of a contralateral steroid-treated sponge.

A further cohort of wild-type mice underwent adrenalectomy or sham surgery as described (29) at the time of implantation of untreated sponges. These mice were then maintained on 0.9% saline in place of drinking water.

Twenty days after implantation (10), mice were killed, sponges were excised, and inserts were removed. Sponges were bisected; one half was fixed in 10% formalin and embedded in paraffin wax. Sections (8 μm) were stained with hematoxylin/eosin for identification of blood vessels, as described in ref. 30. The second half of the sponge was weighed, homogenized in 2 ml of sterile PBS at 4°C, and centrifuged (2,000 $\times$ *g* for 30 min). Steroids were extracted from the supernatant by using ethyl acetate and cortisol quantified by using a specific RIA (Amersham Pharmacia Biotech). Sponge vessel density was determined by using the mean of triplicate Chalkley counts on two sections per sponge (31, 32).

Chronic Coronary Artery Ligation. Wild-type and 11βHSD-1 $-/-$ mice were anesthetized with an i.p. injection of xylazine (0.018 mg/kg), ketamine (100 mg/kg), and atropine (600 mcg/kg) (33). Surgery was performed as described in ref. 34. Briefly, after endotracheal intubation and mechanical ventilation (MiniVent, Harvard Apparatus, Holliston, MA), superficial tissues were dissected, an incision was made in the fourth intercostal space, the pericardium was divided, and the left main descending artery was ligated with 6.0 prolene suture (Ethicon). In sham operated animals, the suture was not ligated. The thoracic wall was closed by layered suturing; the skin was stitched with a continuous suture by using 5-0 Mersilk with a 10-mm 3/8c round-bodied needle (Ethicon). At the time of surgery completion, animals received i.p. atipamazole (5 mg/kg) and s.c. buprenorphine (0.05 mg/kg).

A further cohort of wild-type mice received a s.c. 10 mg implant (28) containing either vehicle or 5.25 mg of RU38486 1 week before coronary artery surgery.

In preliminary experiments, mice were killed on days 1, 3, 5, 7, and 14 after surgery by cervical dislocation. The angiogenic response was well established 7 days after infarction (see Fig. 3*B*), so this interval was selected for comparisons between the groups above. Excised hearts and surgical thoracotomy wounds were fixed in 10% formalin, paraffin embedded, and sectioned at 8 μm. Sections were stained with an anti-von Willebrand factor antibody (DakoCytomation, Cambridgeshire, U.K.) to label endothelial cells and quantify angiogenesis. Hematoxylin and eosin was used to stain sections from hearts collected at day 7 after coronary ligation to measure the area of the left ventricle affected by infarction.

Quantification of vessels within the myocardium was achieved by counting large- and medium-sized vessels as described in ref. 35. Vessels were identified at $\times$400 magnification (Zeiss) in vWF (36–38) stained sections. Counting (39) was performed in the four most vascular fields (two endocardial and two epicardial) by using a 0.0625-mm^2 reticule; the borders of the reticule were within the infarct. The area of left ventricle affected by infarction was determined as a percentage of left ventricular wall area (34) and measured at direct light microscopy; images were captured by using a Research Systems (Imaging Research, St. Catherine's, ON, Canada) photometric camera and analyzed by using in-house scripts.

Wound vessel density was determined in the dermis of vWF-stained sections at $\times$250 light microscopy by using the mean of triplicate Chalkley counts on two sections per wound (31).

Statistics. Data are mean $\pm$ SEM. Comparisons were made by ANOVA with least squares difference post hoc tests. Vessel quantification was performed by investigators "blinded" to the origin of the sections. Interassay and intraassay coefficients of variation in wild-type mice were 17% ($n = 32$) and 22% ($n = 18$), respectively, for vessel number in aortic rings after 7 days in culture; 12% ($n = 6$) and 12% ($n = 6$) for vessel density in sponge implants; 19% ($n = 11$) and 10% ($n = 11$) in day-7 infarcts; and 7% ($n = 4$) and 12% ($n = 4$) for day-7 wounds.

Results

Effects of Glucocorticoids and 11βHSD-1 on Angiogenesis *in Vitro* in Aortic Rings. Both corticosterone and 11-dehydrocorticosterone inhibited angiogenesis in wild-type mouse vessels across a range of physiological concentrations (Fig. 1*B*). The angiostatic effect is mediated by glucocorticoid receptors because it was prevented by the antagonist RU38486 (which has no effect in the absence of steroid) but not by the mineralocorticoid receptor antagonist spironolactone (Fig. 1*C*).

Measurement of relevant product generation confirmed both 11β-reductase (0.65 $\pm$ 0.24 pmol/mg) and 11β-dehydrogenase (0.66 $\pm$ 0.28 pmol/mg) activities in aortic rings with similar conversion rates as in positive controls, liver for 11βHSD-1 (0.18 $\pm$ 0.03 pmol/mg), and kidney for 11βHSD-2 (2.13 $\pm$ 1.65 pmol/mg). Pharmacological inhibition of 11βHSDs in aortic rings was achieved with the nonselective inhibitor carbenoxolone (10^{-6}M), which had no direct effect and prevented the antiangiogenic effect of 11-dehydrocorticosterone but not corticosterone (Fig. 1*Di*).

To confirm the role of 11βHSD-1, aortic rings were obtained from homozygous 11βHSD-1 null ($-/-$) mice congenic on a C57Bl6J genetic background (26) and C57Bl6J controls. Angiogenesis in aortic rings from 11βHSD-1 $-/-$ mice was similar to that in wild-type controls in the absence of steroid and inhibited to a similar degree by corticosterone. However, 11-dehydrocorticosterone did not inhibit angiogenesis in vessels from 11βHSD-1 $-/-$ mice (Fig. 1*Dii*).

Effect of Endogenous Glucocorticoids and 11βHSD-1 on Angiogenesis in s.c. Sponge Implants *in Vivo*. Placebo-impregnated sponges excised after 20 days (10) were red on gross inspection with a lace-like covering of blood vessels. At histology, there was an inflammatory infiltrate and an abundance of blood vessels (Fig. 2*Ai*). Sponges from adrenalectomized animals and sponges impregnated with the glucocorticoid receptor antagonist RU38486 both exhibited enhanced angiogenesis (Fig. 2*Bi*) in wild-type mice.

To test the effects of 11-hydroxy and 11-keto-glucocorticoids we used the "human" steroids cortisol and cortisone, which allowed measurement of steroid concentrations within the sponge independently of endogenous murine corticosterone and 11-dehydrocorticosterone (Table 1). In wild-type C57Bl6J mice, both cortisol and cortisone inhibited angiogenesis *in vivo* (Fig. 2 *Aii* and *Bii*). In 11βHSD-1 null mice, angiogenesis was increased in placebo-impregnated sponges. Impregnation with cortisol produced similar cortisol concentrations in wild-type and 11βHSD-1 null mice (Table 1) and inhibited angiogenesis to a similar degree (Fig. 2*Bii*). However, impregnation with cortisone, in contrast with its effects in wild-type controls, did not elevate sponge cortisol concentrations in 11βHSD-1 null mice (Table 1) and did not inhibit angiogenesis (Fig. 2*Bii*).

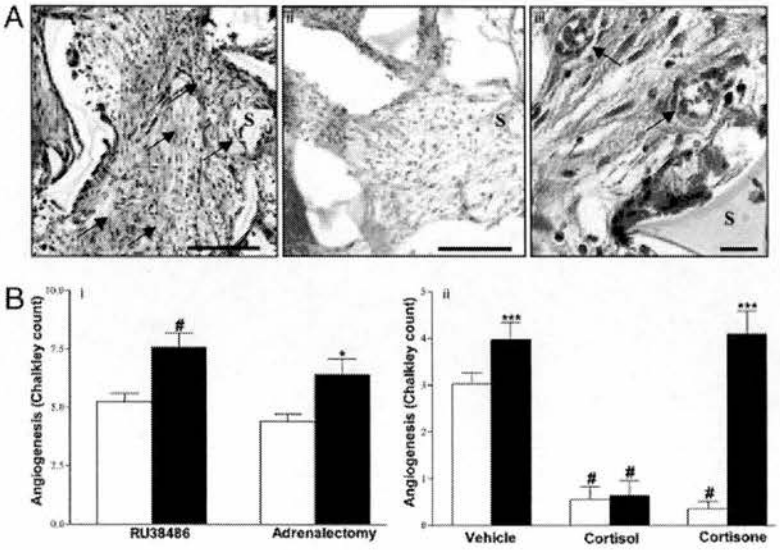

Fig. 2. Angiogenesis in s.c. implanted sponges. (*A*) Light microscopy of hematoxylin/eosin stained sponge 8-μm sections from wild-type mice: vehicle (*i*) and cortisol-treated (*ii*) sponge (Scale bar: 400 μm) and vehicle-treated sponge at high power (*iii*) (Scale bar: 50 μm.) Sponges were covered with a fibroblast-rich fibrous coat and were infiltrated with inflammatory neutrophils and lymphocytes. Placebo-treated sponges alone were also infiltrated with an organized matrix and an abundance of blood vessels (black arrows). S denotes sponge matrix. (*Bi*) Sponges from C57Bl6J wild-type ($n = 6$) mice. Exposure to RU38486 or adrenalectomy (filled bars) compared with placebo or sham surgery (open bars). Results are mean ± SEM. #, $P < 0.01$ versus vehicle; *, $P < 0.02$ versus sham. New vessel formation was greater in RU38486-impregnated sponges or sponges from adrenalectomized mice versus their relevant controls. (*Bii*) Sponges from C57Bl6J wild-type (open bars, $n = 12$) or 11βHSD1 $-/-$ (filled bars, $n = 6$) mice with and without glucocorticoids. Results are mean ± SEM. #, $P < 0.001$ versus corresponding vehicle. ***, $P < 0.001$ for differences between wild type and 11βHSD1 $-/-$. Placebo-impregnated sponges exhibited an increased angiogenic response in 11βHSD1 $-/-$ compared to wild-type mice. Cortisol inhibited angiogenesis in both strains, but cortisone inhibited angiogenesis only in wild-type mice.

Effect of Endogenous Glucocorticoids and 11βHSD-1 on Myocardial Revascularization After Coronary Artery Ligation. At day 7, the proportional area of the left ventricular myocardium affected by coronary artery ligation was similar in all treatment groups (41.8 ± 6.2% in vehicle versus 45.5 ± 4.8% in RU38486 and 44.2 ± 3.4% in wild types versus 44.2 ± 2.6% in 11βHSD-1$^{-/-}$). RU38486 increased angiogenesis in the left ventricle after infarction in wild-type mice (Fig. 3*Bii*).

There was no difference in myocardial vascularity between sham-operated wild-type and 11βHSD-1 null mice. In contrast, 7 days after coronary artery ligation, 11βHSD-1 null mice exhibited enhanced revascularization in the infarcted myocardium (Fig. 3*Bii*),

Effect of Endogenous Glucocorticoids and 11βHSD-1 on Angiogenesis in Cutaneous Surgical Wounds. New vessel formation was examined in cutaneous surgical wounds in mice that underwent thoracotomy for the coronary artery ligation studies (Fig. 3*c*). The dermal angiogenic response was greater in RU38486-treated mice (4.8 ± 0.29 Chalkley count versus vehicle 3.5 ± 0.21; $P < 0.01$) and in 11βHSD-1 null mice (5.1 ± 0.27 Chalkley count; $P < 0.01$) in comparison to wild-type controls (3.5 ± 0.25 Chalkley count).

Discussion

Folkman *et al.* described the angiostatic effects of pharmacological glucocorticoids *in vitro* >20 years ago (11), and these effects have been confirmed *in vivo* (3, 10). Here, we show that the angiostatic effect occurs at physiological concentrations of glucocorticoids and is mediated by glucocorticoid receptors, and that endogenous glucocorticoids tonically repress angiogenic responses. Moreover, we show that 11βHSD1, by regenerating active glucocorticoids locally, amplifies the angiostatic effect of glucocorticoids and, thereby, constrains the angiogenic response after ischemia and injury.

These observations raise the intriguing possibility that local variations in cortisol levels or in tissue sensitivity to cortisol are key determinants of angiogenesis in disease. It is well recognized that, in Cushing's syndrome, glucocorticoid excess is associated with impaired wound healing (40). More recently, we showed that exogenous glucocorticoid therapy is associated not only with increased incidence of myocardial infarction but also with an unexpected increase in prevalence of heart failure (41, 42), suggesting an impact on the outcome and the incidence of cardiovascular disease. More subtle variations in cortisol secretion and action, including variations in responses to stress, have been described in many populations and have been related to risk factors for occlusive vascular disease, mood, development in early life, gender, age, etc. (43–45). We now suggest that effects of cortisol on angiogenesis could explain the links between these quantitative traits in the population and the health outcomes from vascular disease and, perhaps, from other diseases involving angiogenesis, including neoplasia. If so, then therapies, which reduce glucocorticoid action within ischemic tissue, may be valuable in improving collateral perfusion. This result cannot be achieved safely with systemic antiglucocorticoid therapy that is likely to lead to Addisonian crisis after a severe stressor such as myocardial infarction. The role of 11βHSD-1 described here offers an opportunity for tissue-specific targeting of therapy.

We described the presence of 11βHSD-1 in the vessel wall >10 years ago (16), but its importance has remained obscure. The observations that nonselective 11βHSD inhibitors influence vascular tone (27, 46) can be attributed to effects on the 11βHSD-2 isozyme that catalyses inactivation of glucocorticoids within endothelial cells (15, 22). Here, we show that regeneration of glucocorticoids by 11βHSD-1 in isolated aortae amplifies their angiostatic effect. We found no evidence that dehydrogenase 11βHSD-2 influences angiogenesis *in vitro* because the nonselective 11βHSD inhibitor carbenoxolone did not potentiate the angiostatic effect of corticosterone.

In vivo 11βHSD-1 null mice have no obvious difference in vascular structure in healthy tissues. Normal vascular development occurs in other models of altered angiogenesis in

Table 1. Cortisol concentration in sponge homogenates from wild-type and 11βHSD1 $-/-$ homozygous null mice

Strain	Steroid impregnated	Cortisol level (ng/g sponge)	
		Ipsilateral steroid-treated sponge	Contralateral placebo-treated sponge
Wild type	Cortisol	4,271 ± 186#	161 ± 18
	Cortisone	295 ± 25#**	98 ± 19
11βHSD-1 $-/-$	Cortisol	3,775 ± 1,703#	135 ± 46
	Cortisone	87 ± 11	90 ± 30

Results are mean ± SEM for $n = 3$–6 experiments. #, $P < 0.01$ versus contralateral placebo. **, $P < 0.01$ for comparison of wild type and 11βHSD1 $-/-$.

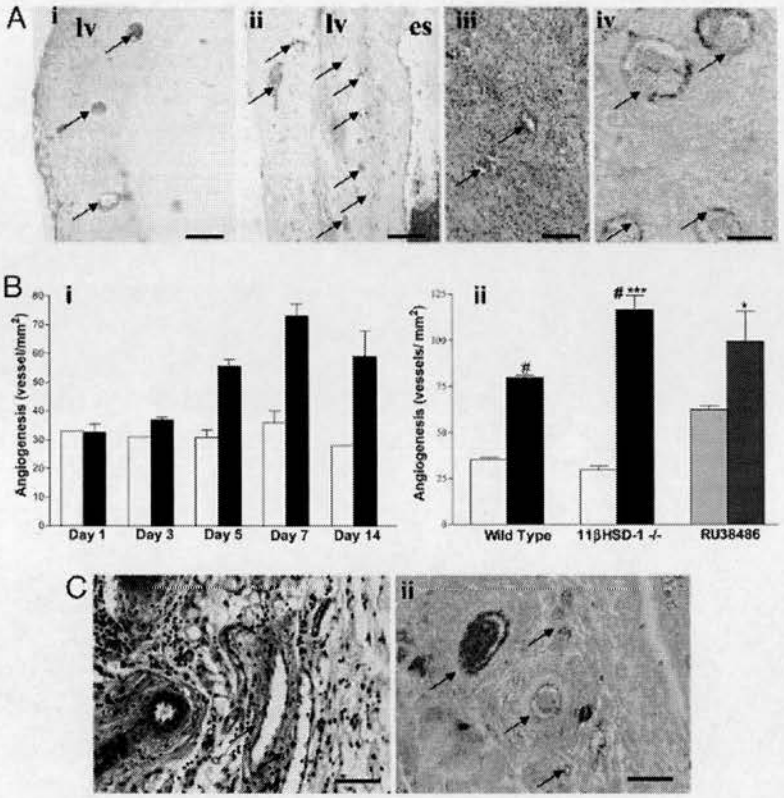

Fig. 3. Effect of injury on angiogenesis in mouse myocardium and skin. (*A*) Light microscopy (×50) of anti-von Willebrand factor immunostaining with fast red chromogen substrate in day 7 wild-type sham (*i*) and infarcted (*ii*) hearts. Scattered medium and large vessels were detected in sham hearts. In contrast, many more vessels were observed in the healing myocardium after infarction. Black arrows, vessels; lv, left ventricle, es, endocardial surface. (Scale bar: 100 μm.) (*iii*) Medium power (×100) light microscopy of hematoxylin/eosin staining in day-7 wild-type infarcted heart. (Scale bar: 100 μm.) (*iv*) High power (×400) light microscopy of anti-von Willebrand immunostaining in day-7 wild-type infarcted heart; (Scale bar: 100 μm.) The vascularity of the infarcted myocardium was increased and multiple vessels containing erythrocytes were observed (black arrows indicate vessels). (*Bi*) Vascularity of myocardium of wild-type mouse hearts after ligation (filled bars, n = 3–11) or sham surgery (open bars, n = 1–6). Sham-operated animals show a constant vascularity in contrast to CCL animals in which vessel counts increase with time, achieving a maximum at day 7. (*Bii*) Day-7 hearts from wild-type and 11βHSD1 $-/-$ mice. Ligation (filled bars) in wild-type and 11βHSD1 $-/-$ increased angiogenesis in comparison to sham (open bars) (wild type, n = 6 sham and n = 11 ligations; 11βHSD1$-/-$, n = 5 sham and n = 10 ligations). Ligations in mice that received RU38486 (dark gray bars, n = 6) induced greater myocardial angiogenesis in comparison to vehicle-treated ligated controls (light gray bars, n = 3). Results are mean ± SEM. #, $P < 0.001$ versus corresponding sham. ***, $P < 0.001$ for differences between wild-type and 11HSD1 $-/-$.*, $P < 0.02$ for differences between coronary artery ligated wild-type mice treated with RU38486 or vehicle. (*C*) Identification of blood vessels in 7-day-old cutaneous wounds from wild-type mice stained with hematoxylin/eosin (*i*) or an antibody against von Willebrand factor (*ii*). (Magnification: ×400; scale bars: 100 μm.)

which the abnormality is apparent only in adult pathology (47), thus reflecting the distinct pathways underlying vasculogenesis and adult angiogenesis. However, when angiogenesis is stimulated in adult mice, we found that 11βHSD-1 amplifies the angiostatic effect of endogenous glucocorticoids. In s.c. sponge implants, this effect is local, rather than systemic, because angiogenesis in contralateral sponges was unaffected. Moreover, cortisol concentrations in the sponges were lower after impregnation with cortisone than with cortisol, suggesting that it is the generation of cortisol locally within the cells that express 11β-HSD1, rather than levels of cortisol in the interstitial fluid of the sponge, which determines the angiostatic effect. Finally, the relevance of 11βHSD-1 was confirmed by the demonstration that 11βHSD-1 null mice exhibit greater angiogenic responses in wounds and infarcted myocardium. In these studies, immunohistochemical localization of vWF enabled quantification of large- and medium-sized vessels (35) but not the entire population of endothelial cells in a section. Thus, all of the vessels included in the quantification are likely to be functional.

It is possible that these observations reflect 11βHSD-1 activity either within the vessel wall or in the inflammatory infiltrate that accompanies angiogenesis in all of these *in vivo* models. 11βHSD-1 is expressed in macrophages (48), and regeneration of glucocorticoids enhances phagocytosis of apoptotic neutrophils (49), hence absence of 11βHSD-1 may confer a prolonged and enhanced acute inflammatory response that, in turn, might stimulate angiogenesis. However, 11βHSD-1 in the inflammatory infiltrate cannot explain the influence of 11βHSD-1 in isolated aortic rings. The findings in the isolated aortic ring model confirm that vessel wall 11βHSD-1 moderates the angiostatic influence of glucocorticoids and confirms that regeneration of active glucocorticoids within vascular smooth muscle cell can inhibit angiogenic processes. Although the *in vivo* models validated the isolated aortic ring findings, it is apparent nonetheless that inflammatory cytokines induce 11βHSD-1 expression in a variety of cell types (13), including in vascular smooth muscle cells (23), so that the contribution of 11βHSD-1 within the vessel wall may be intimately related with the extent of the inflammatory response.

Angiogenesis is crucially dependent on endothelial cells producing key factors such as vascular endothelial growth factor (VEGF) and forming a *de novo* collagen basement membrane to allow structured cell proliferation (24). In the chick chorioallantoic membrane, glucocorticoids alter endothelial cell morphology and collagen production (7, 9). It has also been proposed that glucocorticoid effects are mediated by inhibition of endothelial VEGF transcription and endothelial nitric oxide production (19, 50). However, in keeping with a role for 11βHSD-1, the effect of glucocorticoids may be mediated within vascular smooth muscle, where inhibition of matrix metalloproteinase production (51) may alter the efficacy of endothelium-dependent new vessel formation, and antiproliferative effects (20) may attenuate formation of vessel walls around endothelial cell buds.

In contrast to the established effects of supraphysiological concentrations of glucocorticoids, the influence of endogenous glucocorticoids on angiogenesis has until now remained unclear. The current findings *in vitro* and *in vivo* confirm a physiological role for endogenous glucocorticoids to regulate angiogenesis and highlight the significance of vascular 11βHSD-1 in modulating this effect. The wider relevance of these findings to pathology is illustrated in the models of wound healing and myocardial infarction. These findings may lead to therapeutic approaches to enhance angiogenesis by preventing glucocorticoid action. Although systemic glucocorticoid receptor blockade is unlikely to be successful as a

treatment in the short term (because of adverse effects of preventing the normal cortisol-dependent stress response) or in the long term (because of compensatory activation of the hypothalamic pituitary adrenal axis), the current data suggest that manipulation of local 11βHSD-1 offers a more targeted approach to the blood vessel wall. 11βHSD-1 inhibitors are already being developed for reducing risk factors for cardiovascular disease (45), including in type 2 diabetes mellitus and obesity. These results suggest that pharmacological inhibition of 11βHSD-1 may also be valuable in ischemic heart disease and impaired wound healing.

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Cellular and Molecular Life Sciences

Review

Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function

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Abstract. The ability of glucocorticoids to directly alter arterial function, structure and the inflammatory response to vascular injury may contribute to their well-established link with the development of cardiovascular disease. Recent studies have emphasised the importance of tissue-specific regulation of glucocorticoid availability by the 11β-hydroxysteroid dehydrogenase (11HSD) isozymes, which inter-convert active glucocorticoids and their inactive metabolites. The expression of both type 1 and type 2 11HSDs in the arterial wall suggests that pre-receptor metabolism of glucocorticoids may have a direct impact on vascular physiology. Indeed there is evidence that 11HSDs influence glucocorticoid-mediated changes in vascular contractility, vascular structure, the inflammatory response to injury and the growth of new blood vessels. Hence, inhibition of 11HSD isozymes may provide a novel therapeutic target in vascular disease.

Key words. 11β-Hydroxysteroid dehydrogenase; inflammation; vascular contractility; angiogenesis; cardiovascular disease.

Introduction

There is increasing evidence that direct interaction of glucocorticoids with the vascular wall [1, 2] contributes to their association with increased risk of cardiovascular disease [3, 4]. Certainly, glucocorticoids can interact both with endothelial (EC) and with vascular smooth muscle (VSMC) cells, and furthermore, glucocorticoid-mediated enhancement of vascular contractility has been implicated in the development of hypertension [5]. In addition, glucocorticoids may directly modify new blood vessel formation and vascular lesion development by inhibiting inflammation, proliferation and angiogenic pathways in the arterial wall [6, 7].

Interaction of glucocorticoids with the vasculature is unlikely to be regulated solely by circulating concentrations of these steroids; pre-receptor metabolism within target tissues also has a profound influence on glucocorticoid activity. Such tissue-specific modulation of glucocorticoid activity, regulated by the isozymes of 11β-hydroxysteroid dehydrogenase (11HSD) [8], has a key role, for example, in the development of hypertension, obesity and the metabolic syndrome [9–11]. It is likely that pre-receptor metabolism of glucocorticoids influences steroid action within the vessel wall since both isozymes of 11HSD are expressed in vascular cells [12]. This article reviews the current evidence that vascular 11HSD expression influences glucocorticoid-mediated changes in vascular growth, function, structure and the inflammatory response to vascular injury.

Glucocorticoid signalling in the vascular wall

Glucocorticoids (cortisol in man, corticosterone in rodents) are predominantly synthesised in, and released

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from, the adrenal cortex. Circulating concentrations of these steroids are under the control of the hypothalamic-pituitary-adrenal (HPA) axis, whilst their bioavailability is regulated by interaction with corticosteroid-binding globulin (CBG) and albumin in the plasma. The small proportion of unbound, circulating hormone is able to cross the cell wall and interact with corticosteroid receptors. Classically, glucocorticoids interact with the cytosolic glucocorticoid receptor (GR, or corticosteroid receptor type II). As described below, glucocorticoids may also activate mineralocorticoid receptors (MR, or corticosteroid receptor type I), but this occurs only in a few tissues. GR and MR are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors [13]. Activation of GR results in binding of receptor homodimers to glucocorticoid response elements in target genes, leading to initiation or repression of transcription. There is also increasing evidence that glucocorticoids exert specific, non-genomic actions. Examples exist of rapid glucocorticoid-induced changes to phospholipase A_2 (PLA_2) and phosphoinositide-3-kinase-mediated endothelial nitric-oxide synthase (eNOS) release that are blocked by GR antagonism but not by inhibition of transcription [14, 15]. These non-genomic effects are thought to be mediated by membrane-bound GR [16] (mGR; although the specific signalling pathways associated with these receptors have not been established) or by cytosolic GR (cGR) without requirement for either nuclear translocation or effects on transcription. In the latter case, chaperones or co-chaperones (such as Src) act as signalling components and, therefore, mediators of glucocorticoid-induced effects [17].

Corticosteroid receptors are present in the cells of the vascular wall, supporting the idea that glucocorticoids interact directly with the vasculature. Cytosolic MR and GR have both been demonstrated in freshly isolated vessels [18, 19] and in cultured vascular cells (VSMCs [20, 21] and ECs [22–26]) from a variety of species. The distribution of these receptors may vary with vascular territory, as MR were detected in rabbit aortic and pulmonary VSMCs but not in small arteries [27]. Vascular GR are known to be active as antagonism (with RU38486) blocked dexamethasone-mediated induction of ACE activity in rat aortic ECs [28]. Similarly, activity of MR is demonstrated by their contribution to angiotensin II-induced hypertrophy of VSMCs [29] and aldosterone-induced swelling of ECs [22]. It has not been established whether membrane binding sites for corticosteroids are present, or have a role, in the vascular wall.

The downstream effects of GR activation within the arterial wall, and their influence on cardiovascular risk factors (such as hypertension), are imperfectly understood [5]. Glucocorticoids are essential for maintenance of blood pressure in healthy individuals [1], whilst their ability to increase peripheral vascular resistance in animals devoid of renal mass indicates that a non-renal mechanism contributes to glucocorticoid-induced hypertension [30]. A considerable body of evidence suggests that this non-renal mechanism may involve direct glucocorticoid-mediated alteration of EC and VSMC function [1]. Consequently, regulation of glucocorticoid availability by 11HSDs within the vascular wall may be an important influence on cardiovascular physiology and pathology.

Tissue-specific metabolism of glucocorticoids by 11β-hydroxysteroid dehydrogenases

The 11HSDs, microsomal enzymes of the short-chain alcohol dehydrogenase superfamily [8], interconvert active glucocorticoids and their inert 11-keto forms [31]. Two isozymes, 11HSD1 and 11HSD2, have been identified: 11HSD1 is a low-affinity NADP(H)-dependent, predominant reductase *in vivo*. Dehydrogenase activity of this isozyme is generally not seen in intact cells or organs (including liver [32–34], adipose tissue [35], neurons [36] and vascular smooth muscle [37]); early suggestions of 11HSD1 dehydrogenase activity in vascular smooth muscle [38] are probably attributable to 11HSD2 [37]. 11HSD1 dehydrogenase activity observed in some preparations *in vitro* [39] is probably attributable to release of enzyme from damaged or dying cells, with dissociation from hexose-6-phosphate dehydrogenase, which is thought to maintain the high NADPH concentrations required for reductase activity [40]. 11HSD1 has a Km in the micromolar range for both cortisol and corticosterone [41] and is widely expressed in glucocorticoid-target tissues (including liver, lung, adipose tissue, brain, vascular smooth muscle, skeletal muscle, anterior pituitary, gonads and adrenal cortex [8]), where its role is to amplify local glucocorticoid concentrations [42]. Regulatory control of 11HSD1 is complex, with its synthesis and activity influenced by a variety of factors (such as glucocorticoids [43–45], stress [46, 47], sex steroids [48], growth hormone [49], cytokines [50] and peroxisome proliferator-activated receptor agonists [8]) and its activity driven in the reductase direction through local generation of NADPH by hexose-6-phosphate dehydrogenase [51]. Other factors that may drive 11HSD1 activity in the reductase direction include the cellular environment, cofactor availability, redox potential and substrate concentration.

11HSD2, by contrast, is a high-affinity NAD-dependent, exclusive dehydrogenase, which converts active glucocorticoids into inactive 11-ketosteroids and has a Km for cortisol and corticosterone in the nanomolar range. It is found primarily in mineralocorticoid target tissues, such as the kidney, sweat glands, salivary glands and colon 8], where it is constitutively active and serves to protect MR

from illicit occupation by glucocorticoids. Inhibition of 11HSD2 with liquorice or its derivatives results in glucocorticoid-dependent 'apparent' mineralocorticoid excess and hypertension [52]. Similarly, transgenic disruption of 11HSD2 [9] in mice, or congenital deficiency in man [53], recapitulates the major features of the syndrome of apparent mineralocorticoid excess (SAME). The importance of 11HSD2 in SAME was demonstrated by the description of a defect in cortisol metabolism in children with this syndrome [54]; this was later shown to be the result of mutations in the 11HSD2 gene [55, 56]. 11HSD2 is also expressed in tissues which are not classic MR targets, including the lung, lymph nodes, heart, blood vessel wall and placenta) [57–59]. In the placenta 11HSD2 acts to protect the foetus from excessive exposure to maternal glucocorticoids [60, 61], whereas cardiac 11HSD2 activity may have a role in preventing fibrosis resulting from stimulation of MR by glucocorticoids [62].

The influence of 11HSD isozyme activity on cardiovascular physiology and pathophysiology is well recognised (see Krozowski and Chai for review [63]), but details of the role of 11HSDs within the vessel wall have emerged only recently and remain somewhat uncertain.

Intra-vascular glucocorticoid metabolism

Both isozymes of 11HSD are expressed in the blood vessel wall, suggesting that they could influence vascular function by regulating local availability of active glucocorticoids [1, 64]. The cellular distribution of vascular 11HSD1 and 11HSD2 is not completely clear. Our studies using mouse and rat aorta suggest that 11HSD2 is localised to ECs, whereas 11HSD1 is predominantly in the VSMC (fig. 1) [18, 65]. Others, in contrast, have reported activity of both enzymes in the VSMC [37, 50] and also in the EC [66], it should be noted that the latter investigation [66] demonstrated only 11HSD1 in rat VSMC and indicated that 11HSD1 was the predominant isozyme in the endothelium. Direct comparison of studies is often difficult, given the use of arteries from different species and anatomical locations combined with a variety of techniques for detecting 11HSDs. The balance of the literature suggests that cellular distribution of 11HSD isozymes differs in vessels from distinct anatomical locations and that 11HSD activity increases as artery diameter diminishes; in the rat 11HSD, activity was greater in resistance (mesenteric) arteries than in conduit vessels (aorta) [65] and in the mouse 11β-reductase activity was higher in iliofemoral arteries than in the aorta [A. R. Dover et al., unpublished data]. These variations in cellular distribution and activity suggest that the role of intravascular glucocorticoid metabolism is not the same in all blood vessels.

There is increasing evidence that interconversion of active and inactive glucocorticoids by vascular cells may influence glucocorticoid-mediated modulation of vascular function, structure, growth and inflammation.

Glucocorticoids, 11HSDs and vascular function

Although it is well established that glucocorticoids contribute to maintenance of vascular tone *in vivo,* the mechanisms have been difficult to establish. A variety of interactions contribute to homeostasis, including glucocorticoid-mediated regulation of cardiac output and fluid and electrolyte balance, with salt and water handling modulated both directly [67] and indirectly by influences on the production of angiotensinogen (liver), arginine vasopressin (AVP; hypothalamus) [68] and atrial natriuretic peptide (ANP; cardiac myocytes) [69]. It is apparent, however, that these cardiac and renal effects cannot account totally for the glucocorticoid-mediated increase in blood pressure, and there is evidence that a component of hypertension arises from enhanced contractility of the vascular wall [70–72]. For example, reversal of adrenocorticotophin-dependent hypertension by administration of l-arginine (the substrate for nitric oxide synthase) suggests that nitric oxide deficiency contributes to the elevation of blood pressure [73, 74].

Glucocorticoid-dependent potentiation of noradrenaline- and angiotensin II-mediated vasoconstriction has been at-

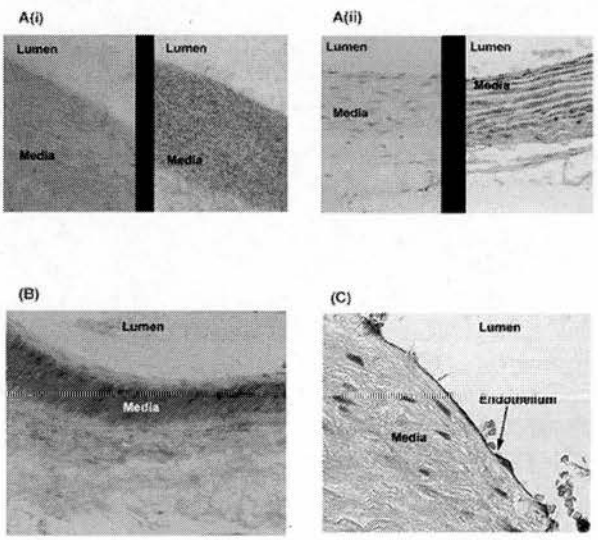

Figure 1. Presence and distribution of 11HSD isozymes in the vascular wall. *In situ* hybridisation (*Ai*) and immunohistochemistry (*Aii*) confirming both expression and activity of 11HSD1 within the rat aortic wall; the enzyme was predominantly localised to medial smooth muscle cells (left-hand panel, sense/control; right hand-panel, antisense/antibody to 11HSD1). Immunohistochemistry demonstrating (*B*) 11HSD1 in rat mesenteric artery smooth muscle and (*C*) 11HSD2 in human intra-renal artery endothelium [unpublished]. Reproduced from [65] with permission. © The Endocrine Society, 1991.

tributed to alterations within the VSMC and the EC (reviewed in Walker and Williams [2] and Ullian [1]). Given that glucocorticoids can act on both MR and GR, the increased contractility observed in many studies may be secondary to increased stimulation of either receptor. Alterations identified within the VSMC (including upregulation of contractile receptors, altered intracellular second messenger activation and modulation of the activity and synthesis of vasoactive substances) result in a direct enhancement of contraction [1]. In contrast, changes in the endothelium can increase contractility in two distinct ways: by increased release of vasoconstrictor compounds (e.g. angiotensin II, endothelin-1 [75, 76]) from the ECs and by impaired endothelium-mediated relaxation. Loss of endothelium-mediated relaxation [77], caused by impaired activity of vasodilators (e.g. prostaglandins, nitric oxide) [78–80], [reduces the ability of the endothelium to modulate contraction.

An alternative mechanism through which glucocorticoids may regulate vascular function is 'foetal programming' of physiological responses [81]. Exposure of the foetus to excess maternal glucocorticoid (either by direct infusion or by inhibition of placental 11HSD2) causes reduced birth weight [82], an outcome associated with increased risk of cardiovascular and metabolic disease in adulthood [83]. Two major causes of low birth weight, maternal dietary restriction and maternal stress, may also be glucocorticoid-dependent [84, 85]. In the ovine foetus, glucocorticoid infusion elevates blood pressure and alters vascular contractility in foetal sheep [86]; this may be significant, as one outcome of foetal programming is elevated blood pressure in adult offspring [87]. However, although enhanced vascular contractility has been demonstrated in rats with programmed hypertension, it is not clear whether this contributes to the elevation of blood pressure [88, 89]. Furthermore, the mechanisms through which pre-natal exposure to excess glucocorticoid programme enhance contractility in adult offspring have not been established.

Influence of 11HSDs on vascular function

In SAME, 11HSD2 deficiency results in sodium retention and severe hypertension, mediated in part by glucocorticoid-dependent activation of MR in the distal nephron [90]. There is, however, a considerable literature to suggest that changes in 11HSD activity within the vascular wall also contribute to elevation of blood pressure. A clear example of this is the demonstration that 11HSD activity is impaired in arteries taken from rat models of hypertension [91–93]. A role for altered vascular function is supported by reports that 11HSD inhibition (with glycyrrhetinic acid) in rats produced an elevation of blood pressure which, whilst mediated by MR activation, was blocked by antagonists of the endothelin-1 system [94, 95]. Moreover, studies of dermal vasoconstriction in patients exposed to liquorice, and in a single individual with SAME [53, 96], demonstrated enhanced cortisol-mediated constriction (fig. 2). The possibility that this is due to changes in glucocorticoid metabolism within the vascular wall, rather than indirect systemic effects of sodium retention, gained further credence with *in vitro* studies which showed that bile acids (e.g. chenodeoxycholic acid), which are endogenous inhibitors of 11HSD [97], pharmacological inhibitors of 11HSD (carbenoxolone, glycyrrhetinic acid) [98, 99] and isozyme selective antisense oligonucleotides [100] alter corticosterone-mediated enhancement of vasoconstriction. Furthermore, 11HSD inhibition (with glycyrrhetinic acid) augmented corticosterone-induced dysfunction in cultured human ECs, indicating both a role for intra-cellular 11HSD and independence from blood pressure elevation *in vivo* [95]. Care is required in interpreting these results, however, as some 11HSD inhibitors can directly alter contractile function by damaging the endothelium [101].

These pharmacological studies have been extended by the use of arteries from 11HSD knockout mice. Aortic function (and blood pressure) are unaltered in 11HSD1$^{-/-}$ mice suggesting that intravascular regeneration of active glucocorticoids has no effect on vascular contractility [102, 103]. This indicates that despite the ability of glucocorticoids to enhance vascular contraction, impaired corticosterone generation in the arterial wall does not re-

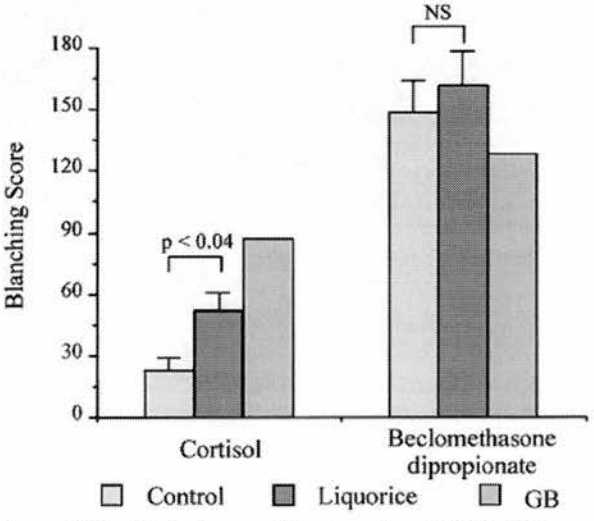

Figure 2. The effect of congenital and acquired 11HSD deficiency on dermal vasoconstrictor sensitivity to cortisol and beclomethasone dipropionate. Inhibition of 11HSD activity with liquorice-enhanced dermal vasoconstriction (measured by skin blanching) in response to cortisol but not to beclomethasone diproprionate. A similar result was obtained in a patient (GB) with the syndrome of apparent mineralocorticoid excess type 1 (11HSD2 deficiency). These data indicate that local regulation of glucocorticoid activity in the vascular wall contributes to contractile tone. Bars are s.e. NS, not significant. Reproduced from [53] with permission. © The Biochemical Society, 1992.

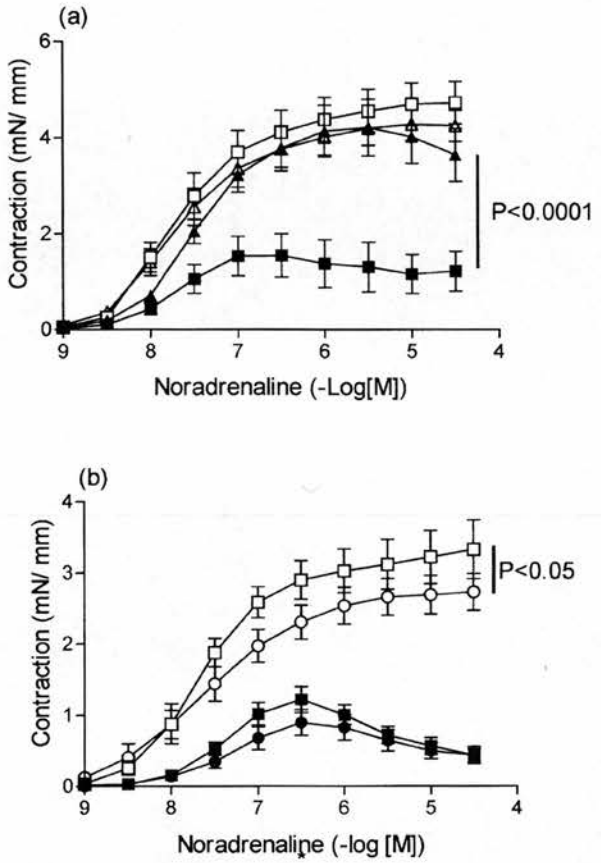

Figure 3. Effect of (*a*) transgenic deletion of 11HSD2 and (*b*) exposure to glucocorticoids on mouse aortic endothelial cell function. In aortic rings isolated from control mice (squares), release of endothelium-derived nitric oxide acts as a physiological antagonist of noradrenaline-mediated contraction; thus, removal of the endothelium (open symbols) results in enhanced contraction. In aortae from 11HSD2$^{-/-}$ mice (*a*; triangles) this ability of the endothelium to modulate contraction has been lost, suggesting glucocorticoid-mediated impairment of endothelial cell function. This is contested, however, by the demonstration that (*b*) *in vitro* incubation (24 h, 37 °C; 10^{-7} M corticosterone) of aortic rings from control animals with glucocorticoids (circles) did not produce a similar endothelial cell dysfunction. Values are mean ± s.e. mean; n=8. Adapted with permission from [103] and [18]. © Lippincott, Williams and Wilkins.

duce contractile function. In contrast, enhanced contractility was demonstrated in thoracic aortae from mice lacking 11HSD2 as a result of impaired endothelium-derived NO activity [103], rather than changes in the VSMC (fig. 3a). This suggests that 11HSD2 activity in the endothelium may serve to protect endothelium-dependent relaxation from the detrimental effects of glucocorticoids. This appears to be consistent with *in vivo* studies which suggest that non-selective inhibitors of both isozymes of 11HSDs, such as liquorice derivatives, potentiate rather than impair the vascular actions of glucocorticoids, suggesting inhibition of inactivation rather than reactivation of steroids within the vessel wall. More recent data, however, suggest that the concept of protection of ECs by 11HSD2 may be an oversimplification. In mouse aortic rings, we could not induce EC dysfunction by incubating isolated rings with glucocorticoids (fig. 3b), even in the absence of 11HSD2 [18]; whether dehydrogenase activity of 11HSD1 [38] served to protect the endothelium is not clear, although our data suggest that 11HSD1 does not act as a dehydrogenase in intact arteries. This raises the possibility that *in vivo* differences in vascular function in 11HSD2 knockout mice are dependent on indirect mechanisms, e.g. related to hypertension or sodium retention. Cell-specific manipulation of 11HSDs would be the most attractive means to dissect this biology further, but has yet to be reported for vascular cells.

Glucocorticoids, 11HSDs and vascular inflammation

Whereas studies in transgenic mice have suggested that 11HSD2 activity may influence vascular function, whilst 11HSD1 does not, a much clearer role for 11HSD1 has been identified in regulation of vascular inflammation. The anti-inflammatory and immunosuppressive effects of glucocorticoids, which account for their most common therapeutic applications, are due to GR-mediated interactions with blood vessels, inflammatory cells and mediators of inflammatory responses [104]. For example, glucocorticoids decrease expression of adhesion factors, cytokines and chemokines, and so alter the recruitment of immune cells such as neutrophils and macrophages to sites of inflammation. This also results in a decrease in leukocyte activation and proliferation. Furthermore, the glucocorticoid receptor mediates lymphocyte apoptosis [105] and suppresses the synthesis of inflammatory mediators (e.g. prostanoids), and hydrocortisone stimulates the synthesis of anti-inflammatory mediators (e.g. lipocortins) [106]. Glucocorticoids, but not mineralocorticoids, can also promote the phagocytosis of apoptotic leukocytes [107], and so contribute to the resolution of inflammation. The expression of 11HSD1 in VSMCs [50] and in activated macrophages [108] suggests that generation of glucocorticoid within these cells may contribute to regulation of inflammation.

Influence of 11HSDs on vascular inflammation

The demonstration that pro-inflammatory cytokines selectively upregulate 11HSD1 activity in human VSMCs suggests that glucocorticoid generation within the vascular wall provides a mechanism for local feedback regulation of inflammation [50]. However, this has not been examined *in vivo*. The ability of inflammatory mediators to regulate 11HSD activity in VSMCs may be dependent upon the phenotypic state of the cells (with enzyme ac-

tivity upregulated in actively proliferating, but not in quiescent, cells), the anatomical origin of a particular vessel, the prevailing local glucocorticoid concentrations and the modulation of the inflammatory response by neighbouring tissues [A. R. Dover et al., unpublished data]. Further work is required to clarify the significance of cytokine-mediated regulation of 11HSD1 in arterial cells, particularly given the importance of inflammation in the vascular response to injury [109].

Alternatively, 11HSD1 may regulate inflammation by controlling generation of glucocorticoids within the inflammatory cells. Certainly, the ability of inflammatory cytokines to upregulate 11HSD1 activity in activated human macrophages [108] suggests, as in human VSMC [50], a means of feedback regulation of inflammation within these cells.

Glucocorticoids and vascular remodelling

The term 'vascular remodelling' has been used to cover a range of structural changes in the arterial wall, and its correct definition is the subject of debate (for review see Bund and Lee [110]). In this review, the term 'vascular remodelling' encompasses medial hypertrophy (thickening of the vessel wall caused by increase in cell size) and hyperplasia (thickening of the cell wall caused by an increase in cell number), as well as the intimal remodelling seen in neointimal proliferation and the development of atherosclerotic lesions. It has also been extended to include angiogenic growth of new blood vessels.

The relationship between glucocorticoids and arterial remodelling is well-established; for example, one year following remission, patients with Cushing's syndrome show reduced intimal/medial thickness and increased lumen diameter in the carotid artery [111]. It should be noted, however, that remodelling may be the indirect result of systemic changes (e.g. increased blood pressure) rather than direct interactions of glucocorticoids with the vessel wall. Glucocorticoids may induce vascular remodelling by altering expression of genes for relevant growth factors or by inhibiting processes that modulate growth factor activity. For example, dexamethasone induces a GR-dependent upregulation of endothelin-1 expression [76], and cortisol attenuates the activity of nitric oxide [77] (itself a potent inhibitor of cell growth). Similarly, dexamethasone- and hydrocortisone-mediated increases in ACE activity in VSMCs [112] and ECs [75] may enhance local generation of angiotensin II (a stimulant of VSMC growth both *in vitro* [113] and *in vivo* [114]).

Hypertrophy

Vascular hypertrophy in rats exposed to mineralocorticoids, predominantly deoxycorticosterone acetate, and salt [115] has been attributed to upregulation of the endothelin-1 gene [116]. Similarly, glucocorticoids (dexamethasone, hydrocortisone) have the ability to induce vascular hypertrophy by augmenting the production of [112, 117], and hypertrophic response to [118, 119], angiotensin II. The significance of this hypertrophy is unclear, however, as many investigations that demonstrate enhanced vascular contractility in response to corticosterone involved a duration of exposure that would be insufficient for vascular hypertrophy to occur [120]. Furthermore, glucocorticoid-mediated stimulation of growth in the vascular wall is counterintuitive given that dexamethasone inhibits VSMC growth in culture [121–123] and glucocorticoids prevent neointimal hyperplasia (see below). Thus, the direct influence of glucocorticoids on vascular hypertrophy/hyperplasia is unclear, and any role of local glucocorticoid metabolism by 11HSDs in the process has yet to be investigated.

Neointimal proliferation

The development of neointimal lesions (e.g. in atherosclerosis and in restenosis following revascularisation) is a consequence of an excessive wound healing response in the vessel wall [124, 125]. Vascular injury results in infiltration by inflammatory cells and subsequent migration and proliferation of VSMCs [109]. Consequently, inhibition of either the inflammatory response [126] or VSMC proliferation/migration [127] inhibits lesion development in a variety of models. Since glucocorticoids (dexamethasone) can inhibit inflammation and VSMC proliferation [121–123] and migration [128], it is not surprising that their potential as anti-atherosclerotic [129] and anti-restenotic agents [130] has been investigated [6]. It is also possible, however, that the action of glucocorticoids on the vessel wall is deleterious in patients with vascular disease. For example, given that ACE inhibition limits neointima formation following balloon injury [131, 132], stimulation of ACE activity by dexamethasone [75, 112] could exacerbate lesion development. Also, inhibition of endothelium-derived nitric oxide activity by glucocorticoids could increase both VSMC proliferation and vascular contraction. Further, the systemic effects of glucocorticoids on cardiovascular risk factors (glucose, insulin, lipids and blood pressure) may offset beneficial effects within the vessel wall.

Dexamethasone reduces cholesterol ester accumulation in the aorta [133], and glucocorticoids (dexamethasone, hydrocortisone) inhibit neointimal lesion formation in rats [134, 135], rabbits [136–138] and dogs [139] (with a few contradictory reports [140, 141]). Clinical trials in humans, by contrast, have proved disappointing (with notable exceptions [130]): methylprednisolone did not inhibit restenosis after coronary angioplasty [142] or stent implantation [143], whilst the combination of a glucocor-

ticoid with colchicine increased the risk of coronary aneurysm following stent placement [144]. Discrepancies between clinical studies and animal models could be attributed to species differences or, more probably, to methodological variation (e.g. small sample size; inappropriate patient selection, dose, duration of treatment, route of administration). Exacerbation of lesion development by glucocorticoids could be explained by systemic effects (e.g. weight gain with elevated blood pressure and plasma lipids, which may be more prominent in humans than in other species) or by a net stimulation of vascular cell proliferation. Alternatively, changes in plasma lipids could influence the ability of glucocorticoids to interact with vascular cells. Lipoprotein(a) can downregulate GR gene expression in human VSMCs, thus inhibiting any protective actions of glucocorticoids and, possibly, representing a novel atherogenic mechanism [145].

Angiogenesis

Angiogenesis, in which new blood vessels are formed from an existing vascular network, is a complex process regulated by a balance between counteracting endogenous activators and inhibitors [146]. Physiological angiogenesis is an essential component of reproduction and embryonic development. In postnatal and adult life, it is a discrete process (e.g. in the reproductive tract, wound healing and exercised skeletal muscle) of relatively short duration [147]. In contrast, pathological angiogenesis is usually persistent and unabated and often continues for months or years [147]. Numerous disorders are characterised by excessive angiogenesis, including neoplasia, rheumatoid arthritis and diabetic retinopathy [148]. Consequently, modulation of angiogenesis is regarded as an attractive therapeutic goal in a variety of conditions.

A comprehensive review of the mechanisms of angiogenesis is beyond the scope of this article (for recent reviews see [146, 149]). For the present purposes it is useful to consider angiogenesis to be a stepwise process comprising four distinct phases: (i) basement membrane disintegration, (ii) endothelial cell migration, (iii) channel formation and (iv) maturation. Of the numerous factors that control this process, vascular endothelial cell growth factor (VEGF) is widely considered to be of central importance, since it is crucial for vascular development both in the embryo and in adult tissues and it is EC specific.

Since its first demonstration by Folkman and colleagues, over 20 years ago [150], the ability of glucocorticoids to inhibit angiogenesis has been confirmed *in vitro, in vivo* and in tumour-bearing animals [150]. It was suggested that inhibition of angiogenesis in the rabbit cornea was independent of classical GR and MR activity [151]. For example, 17α-hydroxyprogesterone and tetrahydro-S, which have no glucocorticoid or mineralocorticoid activity, retained an anti-angiogenic capability equivalent to, or greater than, that of hydrocortisone. Taken together, these studies demonstrated a class of steroids for which inhibition of angiogenesis appears to be the principal function and hence were named 'angiostatic steroids.' [151].

Despite considerable research, the mechanisms through which glucocorticoids inhibit angiogenesis have not been identified. Indeed, the role of GR is still controversial, as some of the 'angiostatic steroids' may actually have the ability to stimulate these receptors. For example, we have recently shown that inhibition of angiogenesis by tetrahydrocorticosterone, one of the original angiostatic steroids, is dependent upon GR activation in mouse aortic ring explants [G. R. Small et al., unpublished]. Some indication of mechanism was provided by early studies which demonstrated, using nude mice or the non-anticoagulant hexasaccharide fragment of heparin, that the combination of glucocorticoid and heparin was independent of an immune response and anti-coagulant function, respectively [150]. At present, however, there are still several possible pathways through which glucocorticoids may inhibit angiogenesis: (i) Degradation of extracellular matrix, (ii) modification of cytokine production, (iii) inhibition of protease activity, (iv) impairment of vessel maturation and stabilisation, (v) inhibition of growth factor activity, (vi) inhibition of the arachidonic acid cascade, (vii) inhibition of EC-leukocyte interactions and (viii) non-transcriptional effects. The relative significance of these pathways has not been established.

11HSDs and vascular remodelling

Although the initial focus was on 11HSD2 and vascular function, the most recent work in the field of intra-vascular glucocorticoid metabolism has highlighted novel roles for 11HSD1 in influencing vascular structure and remodelling.

Neointimal remodelling and atherogenesis

The potential link between atherosclerosis and tissue-specific generation of glucocorticoids by 11HSDs has been underlined by recent demonstrations that selective upregulation of 11HSD1 in the adipose produces features of the metabolic syndrome, including central obesity, hypertension and hypertriglyceridaemia [10, 152]. This supports the concept that similarities between the metabolic syndrome and Cushing's syndrome are explained by tissue-specific increases in 11HSD1 activity resulting in tissue-specific elevation of glucocorticoid generation [153]. This link between 11HSD1 activity in glucocorticoid-target tissues and atherosclerotic risk factors is not limited to the adipose, as hepatic overexpression of 11HSD1 also results in elevated blood pressure and dyslipidaemia

[154]. It has been proposed, therefore, that 11HSD1 inhibition may reduce atherogenesis. Very recently, systemic administration of a selective 11HSD1 inhibitor was reported to virtually abolish lipid accumulation in the aorta of atherosclerotic (apolipoprotein $E^{-/-}$) mice. However, inhibition of 11HSD1 in $ApoE^{-/-}$ mice produced only a relatively modest reduction in serum triglycerides and cholesterol [155], suggesting that mechanisms over and above amelioration of systemic cardiovascular risk factors may be responsible. It may be that inhibition of 11HSD1 within the vessel wall or within invading macrophages [156] is crucial, but these mechanisms require further clarification.

11HSD2-dependent protection of MR from inappropriate occupation by glucocorticoids may also influence atherogenesis. The role of MR activation in the pathogenesis of atherosclerosis [157] is demonstrated by aldosterone-induced enhancement of lesion development in atherosclerotic (apolipoprotein $E^{-/-}$) mice (probably by increasing oxidative stress in macrophages and cells of the vascular wall [157, 158]). This potentiation of lesion development by aldosterone, which is largely independent of blood pressure, is attenuated by MR antagonists [157], as is constrictive remodelling following angioplasty [159].

The potential importance of 11HSD activity to the development of atherosclerotic lesions was recently extended by the demonstration that, in addition to glucocorticoid metabolism, 11HSDs catalyse the conversion of the atherogenic oxysterol 7-ketocholesterol to 7β-hydroxycholesterol [160]. 7-Ketocholesterol is present in micromolar concentrations in human atherosclerotic lesions and in nanomolar concentrations in the plasma [161]. Its

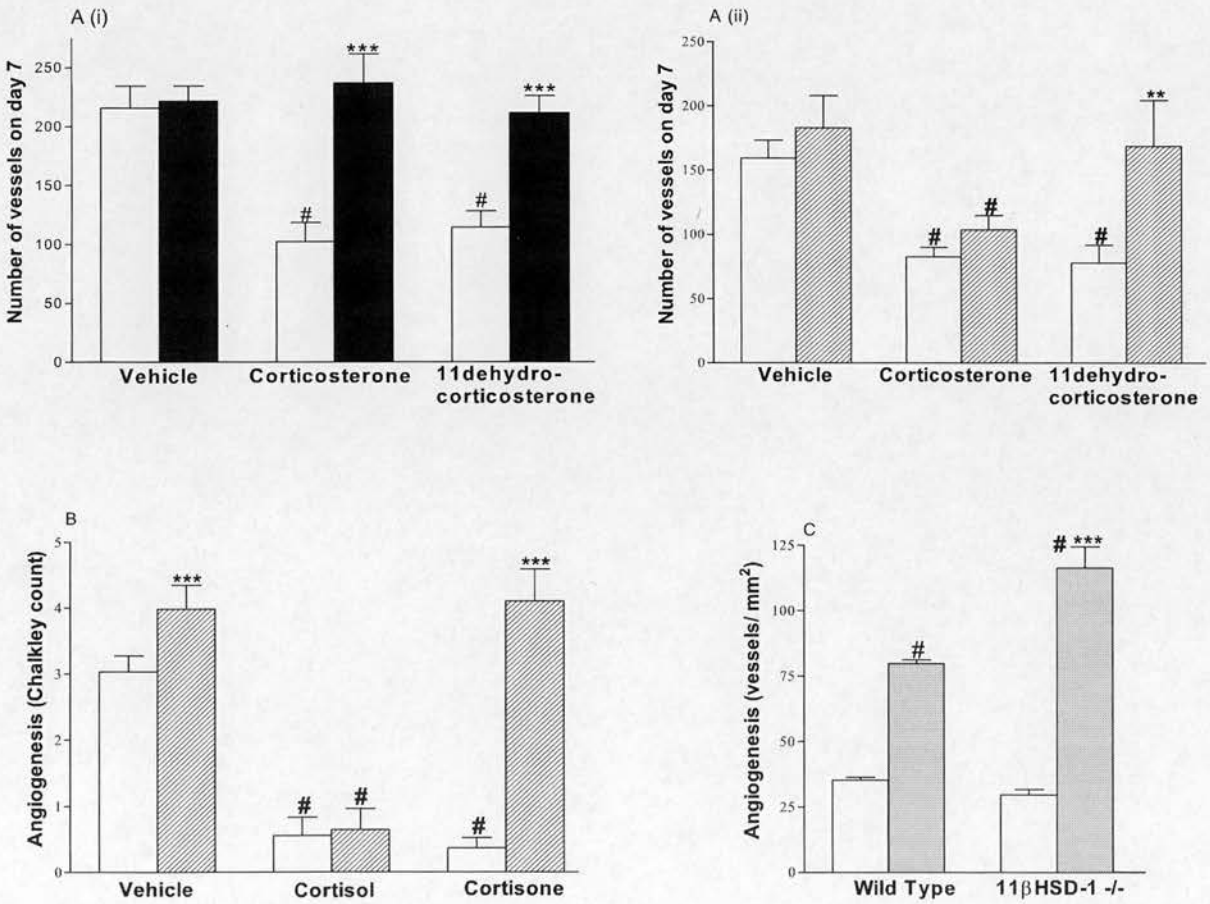

Figure 4. The influence of 11HSD1 on glucocorticoid-mediated angiogenesis. (*A*) In mouse aortic rings cultured in Matrigel, corticosterone and its inactive metabolite 11-dehydrocorticosterone attenuated new vessel growth. Glucocorticoid receptor antagonism (with RU38486, filled columns) abolished the angiostatic response to both compounds (*Ai*), but 11HSD1 deletion (hatched columns) selectively prevented 11-dehydrocorticosterone-mediated angiostasis (*Aii*). Similar results were obtained *in vivo*, using subcutaneous sponge implants (*B*), with 11HSD1 deletion (hatched columns) increasing angiogenesis under basal conditions and abolishing cortisone-, but not cortisol-, mediated inhibition of vessel growth. This mechanism contributed to regulation of myocardial angiogenesis following coronary artery ligation (*C*) with increased vessel growth in $11HSD1^{-/-}$ mice (coronary artery ligation, shaded bars; sham, open bars). #$p < 0.05$ compared with relevant vehicle-treated control; **$p < 0.01$ compared with wild-type mice; ***$p < 0.001$ compared with relevant wild-type control or sham-operated mouse. Reproduced with permission from [168]. © The National Academy of Sciences of the USA, 2005.

association with atherosclerosis is demonstrated in the condition cerebrotendinous xanthomatosis, in which patients who have normal circulating cholesterol levels but increased 7-ketocholesterol develop atherosclerosis prematurely [162]. Conversion of 7-ketocholesterol to 7β-hydroxycholesterol by 11β-HSD1 may represent the rate-limiting step in a clearance pathway: *in vivo* inhibition of 11HSD1 in rats resulted in an accumulation of 7-ketocholesterol in the liver and increased concentrations in the plasma [163]. In addition to these hepatic effects, reduction of 7-ketocholesterol by 11HSD1 within the vascular wall may also be important. 7-Ketocholesterol and 7β-hydroxycholetserol are both toxic to cells of the vascular wall [164] and are potent inhibitors of endothelium-dependent relaxation [165–167]. Consequently, reduction of 7-ketocholesterol, and subsequent clearance of 7β-hydrocholesterol, by protecting the vascular wall from damage may have a role in preventing lesion development.

The influence of 11-HSD activity on angiogenesis

Since inflammatory cytokines can promote angiogenesis, we hypothesised that 11β-HSD1 in the vessel wall may regulate new vessel formation by controlling the local regeneration of active glucocorticoids. This possibly was addressed using a combination of *in vitro*, *in vivo* and pathological models of angiogenesis [168]. Using a model of tube formation from mouse aortic rings cultured in Matrigel [169], we demonstrated (fig. 4A) that angiogenesis was inhibited by physiological concentrations of active glucocorticoid (corticosterone) but also by its inactive metabolite (11-dehydrocorticosterone). Both these responses were blocked by RU38486, but not by spironolactone, indicating GR dependence. However, whereas 11HSD1-inhibition (with carbenoxolone) or deletion (aortic rings from 11HSD1$^{-/-}$ mice) had no effect on the response to corticosterone, they abolished the ability of 11-dehydrocorticosterone to inhibit angiogenesis (fig. 4B). This indicated that 11HSD1-dependent regeneration of active glucocorticoid within the vascular wall regulates new vessel growth. Application of a model of angiogenesis in sub-cutaneous sponge implants, confirmed this role for 11HSD1 *in vivo*, showing that 11HSD1 deletion produced increased angiogenesis in untreated sponges and blocked the ability of cortisone (but not cortisol) to inhibit new vessel formation. The pathophysiological significance of these observations was emphasised in healing cutaneous wounds and in the myocardial response to coronary artery ligation (fig. 4C). In both cases, 11HSD1 deletion resulted in increased angiogenesis, demonstrating that 11HSD1 regulates the growth of new blood vessels in healing tissues.

Altered angiogenesis in 11HSD1$^{-/-}$ [168] mice could, conceivably, be the result of changes in macrophage activity. Given that 11HSD1 is expressed in macrophages [170], and regeneration of glucocorticoids enhances phagocytosis of apoptotic neutrophils [107], absence of 11HSD1 may confer a prolonged and enhanced acute inflammatory response, thus stimulating angiogenesis. The use of *ex vivo* models such as isolated aortic rings cultured in extracellular matrices [171] has made it possible to differentiate between these two intimately related pathways, angiogenesis and inflammation, and specifically address the effects of glucocorticoids on angiogenesis in the absence of a systemic response. This has produced evidence that glucocorticoids regulate angiogenesis by direct interaction with the vessel wall [168].

Conclusions

It is apparent that glucocorticoids have the ability to regulate both the structure and the function of the artery wall, with significant implications for vascular physiology and pathophysiology. Emerging evidence suggests that pre-receptor metabolism of glucocorticoids within vascular ECs and VSMCs provides a mechanism for regulating these interactions. Relatively few studies have addressed the role of intravascular 11HSD activity, and most of those available have focussed on vascular function. There is, however, a growing body of evidence to suggest that 11HSD isozymes within the arterial wall modulate vascular contractility, the angiogenic growth of new blood vessels, and the atherosclerotic process. Whether these isozymes also influence the inflammatory response to vascular injury and the inter-conversion of atherogenic oxysterols in vascular smooth muscle has still to be determined. Further clarification of the role of 11HSDs in vascular cells is likely to increase our understanding of the link between glucocorticoids and a variety of vascular diseases, and to demonstrate their potential as therapeutic targets for treatment of these conditions.

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